INTERNATIONAL SEARCH REPORT

[redacted]

Inte[redacted]nal Application No
P[redacted]S 00/09110

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/29 C12N15/82 C07K14/415 G01N33/68 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EMBL, BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE EMBL [Online] ACCESSION NO: AF090698, 23 September 1998 (1998-09-23) KIM, C.Y., ET AL.: "Oryza sativa elicitor-responsive gene-3 (ERG3) mRNA, complete cds." XP002148594 the whole document & DATABASE STREMBL [Online] ACCESSION NO: O82550, 1 November 1998 (1998-11-01) abstract --- -/--	1,10, 16-18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 September 2000	08.01.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	MADDOX, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE EMBL [Online] ACCESSION NO: AA556184, 3 August 1998 (1998-08-03) ALLONA, I., ET AL.: "39 Loblolly pine N Pinus taeda cDNA clone 2N5F, mRNA sequence." XP002148595 the whole document ---	1,10, 16-18
A	DATABASE DBEST [Online] dbEST Id: 1290863, 22 September 1997 (1997-09-22) SASAKI, T: XP002148596 the whole document & DATABASE EMBL [Online] ACCESSION NO: C73264, 19 September 1997 (1997-09-19) ---	1,10, 16-18
A	XOCONOSTLE-CAZARES BEATRIZ ET AL: "Plant paralog to viral movement protein that potentiates transport of mRNA into the phloem." SCIENCE (WASHINGTON D C), vol. 283, no. 5398, 1 January 1999 (1999-01-01), pages 94-98, XP002148593 ISSN: 0036-8075 the whole document -& DATABASE EMBL [Online] ACCESSION NO: AF079171, 20 January 1999 (1999-01-20) XP002148597 the whole document -& DATABASE EMBL [Online] ACCESSION NO:AF079170, 20 January 1999 (1999-01-20) XP002148598 the whole document -& DATABASE EMBL [Online] ACCESSION NO: U95136, 10 March 1998 (1998-03-10) XP002148599 the whole document -& DATABASE EMBL [Online] ACCESSION NO: U95135, 10 March 1998 (1998-03-10) XP002148600 the whole document -& DATABASE EMBL [Online] ACCESSION NO: U64437, 23 August 1996 (1996-08-23) XP002148601 the whole document --- -/--	1,10, 16-18

INTERNATIONAL SEARCH REPORT

[redacted]

Inter[redacted]nal Application No
P[redacted]S 00/09110

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 20470 A (UNIV CALIFORNIA) 12 June 1997 (1997-06-12) the whole document ---	1-22
A	WO 97 07217 A (UNIV FLORIDA) 27 February 1997 (1997-02-27) the whole document ---	
A	BECK D L ET AL: "DISRUPTION OF VIRUS MOVEMENT CONFERS BROAD-SPECTRUM RESISTANCE AGAINST SYSTEMIC INFECTION BY PLANT VIRUSES WITH A TRIPLE GENE BLOCK" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 91, 1 October 1994 (1994-10-01), pages 10310-10314, XP000617721 ISSN: 0027-8424 the whole document -----	

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PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/29, 15/82, C07K 14/415, G01N 33/68, C12Q 1/68 — A2

(11) International Publication Number: **WO 00/60088**

(43) International Publication Date: 12 October 2000 (12.10.00)

(21) International Application Number: PCT/US00/09110

(22) International Filing Date: 6 April 2000 (06.04.00)

(30) Priority Data:
60/128,092 7 April 1999 (07.04.99) US

(71) Applicant *(for all designated States except US)*: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: KREBBERS, Enno [US/US]; 2318 Sconset Road, Ardentown, DE 19810 (US). WENG, Zude [CN/US]; Apartment 1B, 9122 Lincoln Drive, Des Plaines, IL 60016 (US). CAHOON, Rebecca, E. [US/US]; 2331 West 18th Street, Wilmington, DE 19806 (US).

(74) Agent: GEIGER, Kathleen, W.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AE, AL, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: PLANT VIRAL MOVEMENT PROTEIN GENES

(57) Abstract

This invention relates to an isolated nucleic acid fragment encoding a viral movement protein. The invention also relates to the construction of a chimeric gene encoding all or a substantial portion of the viral movement protein, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the viral movement protein in a transformed host cell.

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WORLD INTELLECTUAL PROPERTY ORGANIZATION
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(51) International Patent Classification 7 :
C12N 15/29, 15/82, C07K 14/415, G01N 33/68, C12Q 1/68

A2

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(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KREBBERS, Enno [US/US]; 2318 Sconset Road, Ardentown, DE 19810 (US). WENG, Zude [CN/US]; Apartment 1B, 9122 Lincoln Drive, Des Plaines, IL 60016 (US). CAHOON, Rebecca, E. [US/US]; 2331 West 18th Street, Wilmington, DE 19806 (US).

(74) Agent: GEIGER, Kathleen, W.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AE, AL, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: PLANT VIRAL MOVEMENT PROTEIN GENES

(57) Abstract

This invention relates to an isolated nucleic acid fragment encoding a viral movement protein. The invention also relates to the construction of a chimeric gene encoding all or a substantial portion of the viral movement protein, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the viral movement protein in a transformed host cell.

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NV

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 23 November 2000 (23.11.00)	
International application No. PCT/US00/09110	**Applicant's or agent's file reference** BB1344 PCT
International filing date (day/month/year) 06 April 2000 (06.04.00)	**Priority date** (day/month/year) 07 April 1999 (07.04.99)
Applicant KREBBERS, Enno et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   30 October 2000 (30.10.00)

   [ ] in a notice effecting later election filed with the International Bureau on:

   _____

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer R. E. Stoffel
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

INTERNATIONAL SEARCH REPORT

ational application No.
PCT/US 00/09110

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-22 all partially

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-22 all partially

Vitis polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:1 and 2, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

2. Claims: 1-22 all partially

Corn polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:3 and 4, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

3. Claims: 1-22 all partially

Corn polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:5 and 6, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

4. Claims: 1-22 all partially

Corn polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:15 and 16, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

5. Claim : 23 partially

Corn polynucleotide sequence encoding a polypeptide of SEQ ID NO:34 and identity as defined in the claim.

6. Claim : 23 partially

Corn polynucleotide sequence encoding a polypeptide of SEQ ID NO:36 and identity as defined in the claim

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

7. Claim : 23 partially

Corn polynucleotide sequence encoding a polypeptide of SEQ ID NO:44 and identity as defined in the claim

8. Claims: 1-22 all partially

Hevea polynucleotide sequences and corresponding polypeptide sequences SEQ ID NOS:7,8,17 and 18, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

9. Claims: 1-23 all partially

Wheat polynucleotide sequences and corresponding polypeptide sequences SEQ ID NOS:9,10,23,24,31,32,37,38,47,48,55 and 56, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

10. Claims: 1-22 all partially

Rice polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:11 and 12, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

11. Claims: 1-22 all partially

Rice polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:25 and 26, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

12. Claims: 1-22 all partially

Rice polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:27 and 28, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

13. Claim : 23 partially

Rice polynucleotide sequence encoding a polypeptide of SEQ ID NO:40 and identity as defined in the claim

14. Claim : 23 partially

Rice polynucleotide sequence encoding a polypeptide of SEQ ID NO:50 and identity as defined in the claim

15. Claim : 23 partially

Rice polynucleotide sequence encoding a polypeptide of SEQ ID NO:52 and identity as defined in the claim

16. Claims: 1-23 all partially

Soybean polynucleotide sequences and corresponding polypeptide sequences SEQ ID NOS:13,14,19-22,29,30, 41,42,45,46,53 and 54, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

INTERNATIONAL SEARCH REPORT

...information on patent family members

International Application No
PCT/US 00/09110

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9720470 A	12-06-1997	AU 1145397 A	27-06-1997
WO 9707217 A	27-02-1997	AU 726197 B	02-11-2000
		AU 6723396 A	12-03-1997
		CA 2229168 A	27-02-1997
		EP 0843728 A	27-05-1998

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
BB1344		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/09110	06/04/2000	07/04/1999

International Patent Classification (IPC) or national classification and IPC
C12N15/29

Applicant
E.I. DU PONT DE NEMOURS AND COMPANY et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
30/10/2000	31.07.2001
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Keller, Y Telephone No. +49 89 2399 7419

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/09110

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-28 as originally filed

Claims, No.:

1-23 as originally filed

Sequence listing part of the description, pages:

1-31, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 1-22 (partially) and 23.

because:

   ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☒ no international search report has been established for the said claims Nos. 1-22 (partially) and 23.

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-15, 18-22
	No:	Claims	16, 17
Inventive step (IS)	Yes:	Claims	19-22

	No:	Claims	1-18
Industrial applicability (IA)	Yes:	Claims	1-22
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: Xonocastel-Cazares B., et al., Science, 1999, vol 283, pages 94-98

1. Due to their broad wording claims 16 and 17 do not meet the requirements of Art. 33(2) PCT. In its broadest sense the wording substance comprising a nucleotide sequence itself comprising the claimed specific sequences encompasses a cell, cell homogenate, etc... naturally containing said nucleic acids

2. D1 discloses the cloning and sequence (fig. 2 legend) of a plant homolog of a viral movement protein from Curcubita maxima (abstract). The corresponding gene is endogenous from plants and does derive from viral RNA (page 95, paragraph 3). D1 teaches also that such genes must be present in other plants e.g rice, maize (page 95 column 2 to page 96, paragraph 1; fig. 2b).

3. D1 is considered as being the closest prior art among the documents cited in the search report.

4. The main differences between D1 and the present application is the plant from which the plant homolog of a viral movement protein is obtained as well as the corresponding sequences.

5. The technical problem to be solved is to clone another plant homolog of a viral movement protein gene.

6. D1 teaches the man skilled in the art that the above mentioned genes have successfully been cloned in Curcubita maxima. Furthermore, D1 teaches that such gene exist in other plants. In view of this latter teaching the skilled person would have tried to clone equivalent genes in other plants and had had reasonable

expectation of success in doing so using standard techniques since such genes have been readily cloned in various plants.
Cloning the plant homolog of a virus movement protein from Vitis represents a mere selection of a particular plant. Such a selection can only be regarded as involving an inventive step if a unexpected or surprising effect for the skilled person can be shown, this is not the case for the present application.
Therefore, claims 1-4 do not meet the requirements of Art 33(3) PCT.

7. The use of a non inventive proteins and/or the corresponding DNA, RNA... in methods, constructs, transformed organisms... well known by person skilled in the art does not involve an inventive step.
Hence, claims 5-18 do not meet the requirements of Art 33(3) PCT

8. The subject-matter of claims 1-15, 18 is not anticipated by the documents cited in the international search report.
Therefore, said claim meets the requirements of Article 33(2) PCT.

9. The subject-matter of claims 19-22 is not anticipated by the documents cited in the international search report, nor does it obviously derive from it.
Thus, said claims meet the requirements of Art. 33(2)(3) PCT.

Re Item VIII

Certain observations on the international application

8. Claims 11-15 do not meet the requirements of Art 6 PCT. The wording "...viral movement protein.." misleads the skilled person as to the real subject-matter for which protection is sought. Said wording is understood by the skilled person as a movement protein of viral origin and not as the plant homolog of a viral movement protein.
Therefore, for sake of clarity "...viral movement protein.." should be reworded "... a plant homolog of a viral movement protein... "

9. Claims 19-22 are not supported by description in a way enabling the skilled person to reproduce the claimed "invention" without an undue burden of experimentation.

Indeed the condition under which a positive selection would be possible are not disclosed neither is the phenotype of a null mutant. It seems that these aforementioned information are not known in the art and thus should be disclosed by the present application in order to enable the reader to reproduce the claimed "invention".

Thus, claims 19-22 do not meet the requirements of Art 5 and 6 PCT.

JAN 24 2001

RECEIVED LMR

JAN 22 2001

PATENT RECORDS CENTER

PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL SEARCHING AUTHORITY

To:
E.I. DU PONT DE NEMOURS AND COMPANY
Legal/Patent Records Center
Attn. ~~GEIGER, Kathleen W.~~
1007 Market Street
Wilmington, Delaware 19898
UNITED STATES OF AMERICA

TMR

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

	Date of mailing *(day/month/year)* 08/01/2001
Applicant's or agent's file reference BB1344 PCT	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US 00/09110	International filing date *(day/month/year)* 06/04/2000
Applicant E.I. DU PONT DE NEMOURS AND COMPANY et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mireille Claudepierre REY NOTED

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

NOTES TO FORM PCT/ISA/220 (continued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments and any accompanying statement, under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the time of filing the amendments (and any statement) with the International Bureau, also file with the International Preliminary Examining Authority a copy of such amendments (and of any statement) and, where required, a translation of such amendments for the procedure before that Authority (see Rules 55.3(a) and 62.2, first sentence). For further information, see the Notes to the demand form (PCT/IPEA/401).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
BB1344 PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 00/09110	06/04/2000	07/04/1999
Applicant		
E.I. DU PONT DE NEMOURS AND COMPANY et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 8 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   ☒ contained in the international application in written form.

   ☒ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readble form.

   ☒ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☒ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☒ **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   ☒ the text is approved as submitted by the applicant.

   ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   ☒ the text is approved as submitted by the applicant.

   ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   ☐ as suggested by the applicant.

   ☐ because the applicant failed to suggest a figure.

   ☐ because this figure better characterizes the invention.

   ☒ None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/09110

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-22 all partially

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-22 all partially

   Vitis polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:1 and 2, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

2. Claims: 1-22 all partially

   Corn polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:3 and 4, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

3. Claims: 1-22 all partially

   Corn polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:5 and 6, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

4. Claims: 1-22 all partially

   Corn polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:15 and 16, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

5. Claim : 23 partially

   Corn polynucleotide sequence encoding a polypeptide of SEQ ID NO:34 and identity as defined in the claim.

6. Claim : 23 partially

   Corn polynucleotide sequence encoding a polypeptide of SEQ ID NO:36 and identity as defined in the claim

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

7. Claim : 23 partially

Corn polynucleotide sequence encoding a polypeptide of SEQ ID NO:44 and identity as defined in the claim

8. Claims: 1-22 all partially

Hevea polynucleotide sequences and corresponding polypeptide sequences SEQ ID NOS:7,8,17 and 18, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

9. Claims: 1-23 all partially

Wheat polynucleotide sequences and corresponding polypeptide sequences SEQ ID NOS:9,10,23,24,31,32,37,38,47,48,55 and 56, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

10. Claims: 1-22 all partially

Rice polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:11 and 12, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

11. Claims: 1-22 all partially

Rice polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:25 and 26, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

12. Claims: 1-22 all partially

Rice polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:27 and 28, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

13. Claim : 23 partially

Rice polynucleotide sequence encoding a polypeptide of SEQ ID NO:40 and identity as defined in the claim

14. Claim : 23 partially

Rice polynucleotide sequence encoding a polypeptide of SEQ ID NO:50 and identity as defined in the claim

15. Claim : 23 partially

Rice polynucleotide sequence encoding a polypeptide of SEQ ID NO:52 and identity as defined in the claim

16. Claims: 1-23 all partially

Soybean polynucleotide sequences and corresponding polypeptide sequences SEQ ID NOS:13,14,19-22,29,30, 41,42,45,46,53 and 54, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]/US 00/09110

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/29 C12N15/82 C07K14/415 G01N33/68 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EMBL, BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE EMBL [Online] ACCESSION NO: AF090698, 23 September 1998 (1998-09-23) KIM, C.Y., ET AL.: "Oryza sativa elicitor-responsive gene-3 (ERG3) mRNA, complete cds." XP002148594 the whole document & DATABASE STREMBL [Online] ACCESSION NO: O82550, 1 November 1998 (1998-11-01) abstract --- -/--	1,10, 16-18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 September 2000	08.01.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	MADDOX, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE EMBL [Online] ACCESSION NO: AA556184, 3 August 1998 (1998-08-03) ALLONA, I., ET AL.: "39 Loblolly pine N Pinus taeda cDNA clone 2N5F, mRNA sequence." XP002148595 the whole document ---	1,10, 16-18
A	DATABASE DBEST [Online] dbEST Id: 1290863, 22 September 1997 (1997-09-22) SASAKI, T: XP002148596 the whole document & DATABASE EMBL [Online] ACCESSION NO: C73264, 19 September 1997 (1997-09-19) ---	1,10, 16-18
A	XOCONOSTLE-CAZARES BEATRIZ ET AL: "Plant paralog to viral movement protein that potentiates transport of mRNA into the phloem." SCIENCE (WASHINGTON D C), vol. 283, no. 5398, 1 January 1999 (1999-01-01), pages 94-98, XP002148593 ISSN: 0036-8075 the whole document -& DATABASE EMBL [Online] ACCESSION NO: AF079171, 20 January 1999 (1999-01-20) XP002148597 the whole document -& DATABASE EMBL [Online] ACCESSION NO:AF079170, 20 January 1999 (1999-01-20) XP002148598 the whole document -& DATABASE EMBL [Online] ACCESSION NO: U95136, 10 March 1998 (1998-03-10) XP002148599 the whole document -& DATABASE EMBL [Online] ACCESSION NO: U95135, 10 March 1998 (1998-03-10) XP002148600 the whole document -& DATABASE EMBL [Online] ACCESSION NO: U64437, 23 August 1996 (1996-08-23) XP002148601 the whole document --- -/--	1,10, 16-18

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]/US 00/09110

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 20470 A (UNIV CALIFORNIA) 12 June 1997 (1997-06-12) the whole document ---	1-22
A	WO 97 07217 A (UNIV FLORIDA) 27 February 1997 (1997-02-27) the whole document ---	
A	BECK D L ET AL: "DISRUPTION OF VIRUS MOVEMENT CONFERS BROAD-SPECTRUM RESISTANCE AGAINST SYSTEMIC INFECTION BY PLANT VIRUSES WITH A TRIPLE GENE BLOCK" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 91, 1 October 1994 (1994-10-01), pages 10310-10314, XP000617721 ISSN: 0027-8424 the whole document -----	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
/US 00/09110

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9720470 A	12-06-1997	AU 1145397 A	27-06-1997
WO 9707217 A	27-02-1997	AU 726197 B	02-11-2000
		AU 6723396 A	12-03-1997
		CA 2229168 A	27-02-1997
		EP 0843728 A	27-05-1998

05

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
BB1344 PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 00/09110	06/04/2000	07/04/1999
Applicant		
E.I. DU PONT DE NEMOURS AND COMPANY et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 8 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. ***Basis of the report***

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   ☒ contained in the international application in written form.

   ☒ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readble form.

   ☒ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☒ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☒ **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   ☒ the text is approved as submitted by the applicant.

   ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   ☒ the text is approved as submitted by the applicant.

   ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   ☐ as suggested by the applicant.

   ☐ because the applicant failed to suggest a figure.

   ☐ because this figure better characterizes the invention.

   ☒ None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/09110

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-22 all partially

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-22 all partially

   Vitis polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:1 and 2, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

2. Claims: 1-22 all partially

   Corn polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:3 and 4, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

3. Claims: 1-22 all partially

   Corn polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:5 and 6, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

4. Claims: 1-22 all partially

   Corn polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:15 and 16, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

5. Claim : 23 partially

   Corn polynucleotide sequence encoding a polypeptide of SEQ ID NO:34 and identity as defined in the claim.

6. Claim : 23 partially

   Corn polynucleotide sequence encoding a polypeptide of SEQ ID NO:36 and identity as defined in the claim

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

7. Claim : 23 partially

Corn polynucleotide sequence encoding a polypeptide of SEQ ID NO:44 and identity as defined in the claim

8. Claims: 1-22 all partially

Hevea polynucleotide sequences and corresponding polypeptide sequences SEQ ID NOS:7,8,17 and 18, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

9. Claims: 1-23 all partially

Wheat polynucleotide sequences and corresponding polypeptide sequences SEQ ID NOS:9,10,23,24,31,32,37,38,47,48,55 and 56, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

10. Claims: 1-22 all partially

Rice polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:11 and 12, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

11. Claims: 1-22 all partially

Rice polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:25 and 26, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

12. Claims: 1-22 all partially

Rice polynucleotide sequence and corresponding polypeptide sequence SEQ ID NOS:27 and 28, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

13. Claim : 23 partially

Rice polynucleotide sequence encoding a polypeptide of SEQ ID NO:40 and identity as defined in the claim

14. Claim : 23 partially

Rice polynucleotide sequence encoding a polypeptide of SEQ ID NO:50 and identity as defined in the claim

15. Claim : 23 partially

Rice polynucleotide sequence encoding a polypeptide of SEQ ID NO:52 and identity as defined in the claim

16. Claims: 1-23 all partially

Soybean polynucleotide sequences and corresponding polypeptide sequences SEQ ID NOS:13,14,19-22,29,30, 41,42,45,46,53 and 54, sequences with identity to said sequences as defined in the claims,chimeric genes, host cells,methods for selecting and obtaining viral movement protein polynucleotides and nucleic acid fragments based on said sequences.

INTERNATIONAL SEARCH REPORT

International Application No
P[redacted]S 00/09110

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/29 C12N15/82 C07K14/415 G01N33/68 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EMBL, BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE EMBL [Online] ACCESSION NO: AF090698, 23 September 1998 (1998-09-23) KIM, C.Y., ET AL.: "Oryza sativa elicitor-responsive gene-3 (ERG3) mRNA, complete cds." XP002148594 the whole document & DATABASE STREMBL [Online] ACCESSION NO: O82550, 1 November 1998 (1998-11-01) abstract --- -/--	1,10, 16-18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
28 September 2000	08. 01. 01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	MADDOX, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE EMBL [Online] ACCESSION NO: AA556184, 3 August 1998 (1998-08-03) ALLONA, I., ET AL.: "39 Loblolly pine N Pinus taeda cDNA clone 2N5F, mRNA sequence." XP002148595 the whole document ---	1,10, 16-18
A	DATABASE DBEST [Online] dbEST Id: 1290863, 22 September 1997 (1997-09-22) SASAKI, T: XP002148596 the whole document & DATABASE EMBL [Online] ACCESSION NO: C73264, 19 September 1997 (1997-09-19) ---	1,10, 16-18
A	XOCONOSTLE-CAZARES BEATRIZ ET AL: "Plant paralog to viral movement protein that potentiates transport of mRNA into the phloem." SCIENCE (WASHINGTON D C), vol. 283, no. 5398, 1 January 1999 (1999-01-01), pages 94-98, XP002148593 ISSN: 0036-8075 the whole document -& DATABASE EMBL [Online] ACCESSION NO: AF079171, 20 January 1999 (1999-01-20) XP002148597 the whole document -& DATABASE EMBL [Online] ACCESSION NO:AF079170, 20 January 1999 (1999-01-20) XP002148598 the whole document -& DATABASE EMBL [Online] ACCESSION NO: U95136, 10 March 1998 (1998-03-10) XP002148599 the whole document -& DATABASE EMBL [Online] ACCESSION NO: U95135, 10 March 1998 (1998-03-10) XP002148600 the whole document -& DATABASE EMBL [Online] ACCESSION NO: U64437, 23 August 1996 (1996-08-23) XP002148601 the whole document --- -/--	1,10, 16-18

INTERNATIONAL SEARCH REPORT

International Application No
P[redacted]S 00/09110

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 20470 A (UNIV CALIFORNIA) 12 June 1997 (1997-06-12) the whole document ---	1-22
A	WO 97 07217 A (UNIV FLORIDA) 27 February 1997 (1997-02-27) the whole document ---	
A	BECK D L ET AL: "DISRUPTION OF VIRUS MOVEMENT CONFERS BROAD-SPECTRUM RESISTANCE AGAINST SYSTEMIC INFECTION BY PLANT VIRUSES WITH A TRIPLE GENE BLOCK" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 91, 1 October 1994 (1994-10-01), pages 10310-10314, XP000617721 ISSN: 0027-8424 the whole document -----	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/09110

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9720470 A	12-06-1997	AU 1145397 A	27-06-1997
WO 9707217 A	27-02-1997	AU 726197 B	02-11-2000
		AU 6723396 A	12-03-1997
		CA 2229168 A	27-02-1997
		EP 0843728 A	27-05-1998

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08/698,461	15 August 1996 (15.08.96)	US

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Published
With international search report.

(54) Title: REGULATION OF PLANT DEVELOPMENT AND PHYSIOLOGY THROUGH PLASMODESMATAL MACROMOLECULAR TRANSPORT OF PROTEINS AND OLIGONUCLEOTIDES

(57) Abstract

Methods and mechanisms for regulation of macromolecular transport between cells in plasmodesmatal communication with one another are disclosed. Tobacco mosaic virus movement protein (TMV-MP) in wild type and mutant forms is shown and used to affect plant size, carbon metabolism and biomass partitioning. Use of a protein to mediate its own cell-to-cell transport through plasmodesmata is illustrated with wild type and mutant forms of KNOTTED protein from the maize homeobox gene *Knotted*.

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REGULATION OF PLANT DEVELOPMENT AND PHYSIOLOGY THROUGH PLASMODESMATAL MACROMOLECULAR TRANSPORT OF PROTEINS AND OLIGONUCLEOTIDES

CROSS REFERENCE TO RELATED APPLICATION:

This is a Continuation-In-Part of Application Serial No. 08/698,461 filed August 15, 1996. This application claims the benefit of U.S. Provisional Application No.: 60/007,915 filed December 4, 1995.

BACKGROUND OF THE INVENTION

1. Field of the Invention:

The present invention relates generally to the field of plant biology. More particularly, the present invention is directed to compositions and methods for use in regulation of plant growth.

This invention was made with Government support under Grant No. DCB-90-16756 and INB-94-06974, awarded by the National Science Foundation, and Grant No. 90-00070, awarded by the United States Department of Agriculture. The Government has certain rights in this invention.

2. Description of the Related Art:

Present strategies for controlling plant developmental and physiological functions rely on traditional genetic approaches, or on biotechnological approaches that lack a

fully refined conceptual foundation. In terms of manipulation of plant resource allocation, the only approach currently available involves the use of genetic breeding for a desired trait; this is recognized as a slow and complex process. Furthermore, current strategies fail to provide any understanding of the underlying molecular events that are involved in the prioritization of resource allocation to the various regions of the plant body.

Partitioning of assimilates in plants is an important and highly regulated process [Wardlaw I. F. (1990) The control of carbon partitioning in plants. *New Phytologist* **116,** 341-381]. It involves regulation of photosynthesis, intracellular and intercellular transport of metabolites, phloem loading and unloading, storage and other interrelated biochemical processes. Partition of assimilates is closely related to the regulation of growth and development, in as much as growth of different plant parts and organs often requires the import of assimilates from elsewhere in the plant. The relationship between root and shoot biomass is an excellent example of regulation of partition of assimilates. Root-to-shoot ratios vary from plant species-to-species, and are influenced by the environment [Geiger D. R. & Servaites J. S. (1991) Carbon allocation and response to stress. In *Response of plants to multiple stresses* (eds. H. A. Mooney, W. E. Winner & E. J. Pell) pp. 103-127. Academic Press, New York; Mooney H. A. & Winner W. E. (1991) Partitioning response of plants to stress. In *Response of plants to multiple stresses* (eds. H. A. Mooney, W. E. Winner & E. J. Pell) pp. 129-141. Academic Press, New York]. Furthermore, this ratio is responsive to water stress and nutrient deficiencies, and it can be manipulated by exogenous hormonal treatments and light quality [Britz S. J. (1990) Photoregulation of root: shoot ratio in soybean seedlings. *Photochemistry and Photobiology* **52,** 151-159; Incoll L. D., Ray J. P. & Jewer P. C. (1990) Do cytokinins act as root to shoot signals? In *Importance of root to shoot communication in the responses to environmental stress,* Monograph 21 (eds.

W. J. Davies & B. Jeffcoat) pp. 185-199. British Society for Plant Growth Regulation, Bristol; Davies W. J. & Zhang J. (1991) Root signals and the regulation of growth and development of plants in drying soil. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**, 55-76; Tolley-Henry L. & Raper C. D. (1991) Soluble carbohydrate allocation to roots, photosynthetic rate of leaves and nitrate assimilation as affected by nitrogen stress and irradiance. *Botanical Gazette* **152**, 23-33].

The finding [Lucas W. J., Olesinski A., Hull R. J., Haudenshield J. S., Deom C. M., Beachy R. N. & Wolf S. (1993) Influence of the tobacco mosaic virus 30-kDa movement protein on carbon metabolism and photosynthate partitioning in transgenic tobacco plants. *Planta* **190**, 88-96] that a significant reduction in biomass partitioning to roots occurs in transgenic tobacco plants that express the tobacco mosaic virus movement protein (TMV-MP) has raised questions as to the possible effects of this protein on the integrated physiology of tobacco plants. It is now well established that the TMV-MP interacts with plasmodesmata to potentiate virus trafficking between cells [Deom C. M., Oliver M. J. & Beachy R. N. (1987) The 30-kDa gene product of tobacco mosaic virus potentiates virus movement. *Science* **237**, 389-394; Wolf S., Deom C. M., Beachy R. N. & Lucas W. J. (1989) Movement protein of tobacco mosaic virus modifies plasmodesmatal size exclusion limit. *Science* **246**, 377-379; Ding B., Haudenshield J. S., Hull R. J., Wolf S., Beachy. R. N. & Lucas W. J. (1992) Secondary plasmodesmata are specific sites of localization of the tobacco mosaic virus movement protein in transgenic tobacco plants. *Plant Cell* **4**, 915-928; Waigmann E., Lucas W. J., Citovsky V. & Zambryski P. (1994) Direct functional assay for tobacco mosaic virus cell-to-cell movement protein and identification of a domain involved in increasing plasmodesmal permeability. *Proc. Natl. Acad. Sci. USA* **91**, 1433-1437]. In transgenic tobacco plants expressing the TMV-MP, the size exclusion limit (SEL) of plasmodesmata connecting the mesophyll and bundle sheath cells was found to

be increased from 800 Da to greater than 9.4 kDa [Wolf et al. 1989, *supra*; Deom C. M., Wolf S., Holt C. A., Lucas W. J. & Beachy R. N. (1991) Altered function of the tobacco mosaic virus movement protein in a hypersensitive host. *Virology* **180**, 251-256; Ding et al. 1992, *supra*]. This observation raised the possibility that dilated plasmodesmata, within such tissues, may enhance symplasmic carbohydrate transport between cells [Lucas W. J. & Wolf S. (1990) Plasmodesmatal function probed using transgenic tobacco plants. In *Recent advances in Phloem transport and assimilate compartmentation* (eds. J. L. Bonnemain, J. Dainty, S. Delrot & W. J. Lucas) pp. 106-115. Ouest Editions, Nantes Cedex; Lucas et al. 1993, *supra*]. However, contrary to this expectation, these transgenic plants exhibited a decrease in translocation of assimilates, from source leaves, during the day [Lucas et al. 1993, *supra*].

Also, in such transgenic plants expressing the TMV-MP, root-to-shoot ratios were significantly smaller, reflecting reduced carbon allocation and translocation to the roots [Lucas et al. 1993, *supra*]. It is thus of great interest that the TMV-MP affects not only the dilation of plasmodesmata and virus trafficking, but also carbohydrate metabolism and resource allocation, as reflected by changes in root-to-shoot ratios.

Similar considerations are involved in understanding the distribution of other plant products, such as sucrose. Sucrose synthesis occurs within the cytosol of tobacco mesophyll cells, but the pathway followed by sucrose during its movement from the site of synthesis to the cells of the phloem remains equivocal. The prevailing view is that solute movement between mesophyll cells occurs via a symplasmic route through plasmodesmata [Giaquinta, R.T. (1983) Phloem loading of sucrose. *Ann. Rev. Plant Physiol.* **34**, 347-387; Tucker, J.E., Mauzerall, D., Tucker, E.B. (1989) Symplastic transport of carbxyfluorescin in staminal hairs of *Setcreasea purpurea* is diffusive and includes loss to the vacuole. *Plant Physiol.* **90**, 1143-1147; Robards, A.W., Lucas, W.J.

(1990) Plasmodesmata. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41**, 369-419]. In many species, however, the actual process involved in loading into the sieve element-companion cell complex may involve an apoplasmic step [van Bel, A.J.E. (1992) Pathway and mechanisms of phloem loading. In: *Carbon partitioning within and between organisms* (eds. Pollock, C.J., Farrar, J.F., Gordon, A.J.) pp. 53-70. BIOS Scientific Publishers, Ltd., Oxford]. Furthermore, it remains to be elucidated whether the loading process constitutes the rate-determining step, or major control site, in the export of recently fixed photosynthate.

Experimental control over plasmodesmal SEL has recently been achieved using expression of viral movement proteins (MPs) in transgenic plants. In transgenic tobacco expressing the MP of tobacco mosaic virus (TMV-MP), this movement protein becomes localized to mesophyll plasmodesmata [Atkins, D., Hull, R., Wells, B., Roberts, K., Moore, P., Beachy, R.N. (1991) The tobacco mosaic virus 30K movement protein in transgenic tobacco plants is localized to plasmodesmata. *J. Gen. Virol.* **72**, 209-211; Ding et al. 1992, *supra*; Moore, P.J., Fenczik, C.A., Deom, C.M., Beachy, R.N. (1992) Developmental changes in plasmodesmata in transgenic tobacco expressing the movement protein of tobacco mosaic virus. *Protoplasma* **170**, 115-127] where it causes a significant increase in plasmodesmal SEL from the control value of approx. 800 Da to greater than 9.4 kDa [Wolf et al. 1989, *supra*]. Photosynthesis and carbon allocation experiments performed on these transgenic tobacco plants revealed that the presence of the TMV-MP resulted in a change in carbon metabolism [Lucas et al. 1993, *supra*]. Although total chlorophyll, net photosynthesis and total leaf proteins were not significantly different between control and TMV-MP plant lines, it was found that, compared with control plants, fully expanded leaves expressing the TMV-MP had unexpectedly high levels of sugars and starch. The level of carbohydrates within these TMV-MP leaves appeared to increase more rapidly during the photoperiod, compared with control leaves, with

the converse occurring during the dark period. In addition, there was a significant difference in biomass distribution between the various plant organs, resulting in lower root-to-shoot ratios in TMV-MP transgenic plants, although, under the growth conditions employed in these studies, the total biomass remained similar in both plant lines [Lucas et al. 1993, *supra*]. This complex influence of the TMV-MP on carbon metabolism was unexpected, since it was anticipated that increasing the plasmodesmal SEL would enhance symplasmic transport of sugars from the mesophyll to the site of phloem loading. If this were the case, sugar levels within the mesophyll tissue of TMV-MP plants should have been lower, not higher, than control values. Furthermore, if plasmodesmal SEL within the mesophyll did not constitute a rate-limiting step on the process of loading, carbon metabolism should have remained unaffected by the TMV-MP. Interpretation of these results was further complicated by the finding that although the TMV-MP was expressed in the vascular tissue, it did not cause an increase in the SEL of the plasmodesmata that interconnect the cells within the vein [Ding et al. 1992, *supra*].

It is an object of the present invention to provide compositions and methods which do not suffer from all of the drawbacks of the heretofore known compositions and methods.

SUMMARY OF THE INVENTION

In accordance with the present invention, there are provided methods and compositions for use in regulating plant metabolism and growth, wherein a plant regulatory composition (as hereinafter defined) is administered in a manner such that plasmodesmal transport of the composition in a predetermined manner is effected. Evidence is provided herein that plant encoded genes have the capacity to traffic via plasmodesmata to influence cell fate. In one example, TMV-MP is shown to interfere with an endogenous signal transduction pathway that involves macromolecular trafficking through plasmodesmata to regulate biomass partitioning

between the source and various sink tissues. In another example, three homeotic proteins encoded by the maize homeobox gene *Knotted-1* and the MADS box genes *deficiens* and *globosa* of Antirrhinum [Sommer H., Nacken W., Beltran P., Huijser P., Pape H., Hansen R., Flor P., Saedler H., Schwartz-Sommer Z. (1991) Properties of *deficiens*, a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus*. *Development Supp* **1**, 169-175; Troebner W., Ramirez L., Motte P., Hue I., Huijser P., Loennig W.-E., Saedler H., Sommer H., Schwartz-Sommer Z. (1992) *GLOBOSA:* A homeotic gene which interacts with *DEFICIENS* in the control of *Antirrhinum* floral organogenesis. *EMBO J.* **11**, 4693-4704] have now been shown to interact with plasmodesmata to mediate in their cell-to-cell transport. In yet another example, the first direct experimental proof that a plant mRNA-encoded protein can mediate in the plasmodesmal transport of itself and its own mRNA such that the mRNA can undergo extensive cell-to-cell movement is provided.

A further example illustrates that plant growth response to light intensity is altered by a viral movement protein. And, in yet a different example, selective cell-to-cell movements of proteins through plasmodesmata are shown to potentiate cellular interactions between cells in adjacent cell layers, such as: between layers of meristematic tissue; and, between vascular tissue cells and cells in adjacent mesophyll and epidermal layers.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention may be better understood with reference to the accompanying drawings, in which:

Figs. 1A, 1B and 1C illustrate diurnal changes in ^{14}C-photosynthates in leaves of TMV-MP transgenic (●) and vector control (O) tobacco plants, experiments being performed on fully expanded source leaves (#5 and 6) (1A and 1B) or on younger, expanding source leaves (#2 and 3) (1C);

Figs. 2A, 2B, 2C, 2D, 2E, 2F, 2G, 2H, 2I and 2J illustrate carbohydrate content and ^{14}C-radioactivity

detected within source leaves of transgenic tobacco plants expressing wild-type TMV-MP (line 277; ■), temperature-sensitive TMV-MP mutant (line 2-72; ≥) and vector control (line 306; ○), with radioactivity detected using a "Rotem" ß counter (Figs. 2A & 2B) and leaf discs analyzed for starch (Figs. 2C & 2D), sucrose (Figs. 2E & 2F), glucose (Figs. 2G & 2H) and fructose (Figs. 2I & 2J); and

Fig. 3 is a schematic diagram illustrating sites where the TMV-MP might interact with the plant's endogenous control network to cause the observed alterations in sugar metabolism and reallocation of photosynthate to yield a reduction of root-to-shoot ratio in transgenic plants expressing the *TMV-MP* gene.

Fig. 4 is a schematic diagram, in conventional single-letter amino acid code, showing the positions of mutations for different alanine scanning mutants of wild type KN1.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The results of research reported herein demonstrate that macromolecular trafficking of proteins and oligonucleotides via plasmodesmata is involved in control of enzyme function and gene expression. Modulation in this intercellular transport pathway can therefore be used to alter plant performance. Such alterations in macromolecular signalling can be used to redirect available resources, such as fixed carbon, nitrogen compounds (including amino acids) and mineral nutrients, as well as foreign compounds, to specific tissues within the plant. This invention provides compositions and methods which enable one skilled in the art to control this macromolecular trafficking and, thereby, control the rate and direction (e.g., to particular organs of the plant) of resource allocation.

For purposes of the present invention, a "plant regulatory composition" comprises at least one active agent which affects the growth and/or metabolism of the plant. The composition may comprise one or more polypeptides and/or oligonucleotides encoding such polypeptides as an active

agent. In accordance with one preferred class of embodiments of the invention, the plant regulatory composition comprises at least one polypeptide and at least one oligonucleotide operatively encoding the polypeptide.

The active agent may comprise an endogenous or exogenous polypeptide or glycoprotein, including both heretofore known products and newly-engineered ones (e.g., mutant forms, fusion or chimeric proteins, etc.). Exemplary classes of products include, but are not limited to, the following: growth factors (e.g., *KNOTTED-1* as discussed in detail herein); herbicides; insecticides; fungicides; agents against nematode infection; compounds which block herbivory; and compositions which enhance endogenous mechanisms involved in the establishment of general systemic acquired resistance in plants.

The range of applicability of the present invention may be better appreciated by consideration of representative types of agents suitable for incorporation into a plant regulatory composition. An example of a compound which blocks herbivory is the molecule systemin, which is a proteinase inhibitor [Ryan C.A. (1992) The search for the proteinase inhibitor-inducing factor, PIIF. *Plant Mol. Biol. Int. J. Mol. Biol. Biochem. Genet. Eng.* **19**, 123-133; Constabel C.P., Bergey D.R., Ryan C.A. (1995) Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. *Proc. Natl. Acad. Sci. USA* **92**, 407-411]. Systemin is a peptide of 18 amino acids [McGurl B., Pearce G., Orozco Cardenas M.L., Ryan C.A. (1992) Structure, expression, and antisense inhibition of the systemin precursor gene. *Science* **255**, 1570-1573; Ryan C.A., McGurl B. (1992) The organization of the prosystemin gene. *Plant Mol. Biol. Int. J. Mol. Biol. Biochem. Genet. Eng.* **20**, 405-409; Pearce G., Johnson S., Ryan C.A. (1993) Structure-activity of deleted and substituted systemin, an 18-amino acid polypeptide inducer of plant defensive genes. *J. Biol. Chem.* **268**, 212-216] that is released when the plant is attacked by chewing insects (or as

a result of physical injury). This small peptide moves into the phloem [Narvaez-Vasquez J., Pearce G., Orozco-Cardenas M.L., Franceschi V.R., Ryan, C.A. (1995) Autoradiographic and biochemical evidence for the systemic translocation of systemin in tomato plants. *Planta* **195**, 593-600] and is thereby transported to regions where growth is taking place. Within these tissues, systemin acts on gene expression to turn on defense genes, including Proteinase Inhibitor I and II [Ryan 1992, *supra*]. Thus, a plant regulatory composition, in the case of this herbivory blocker, would comprise an oligonucleotide coding for: systemin; defense genes such as Proteinase Inhibitor I and II; and, a movement protein specific for movement of its coding oligonucleotide through plasmodesmata.

Modulations in plant growth or metabolism can be effected via regular utilization of transgenic plants which express an endogenous or exogenous gene, from a plant or other source. The creation and use of modified pathogenic genes and/or the development and expression of artificial genes that can mimic or override the functions performed by endogenous plant proteins encoded by the parent gene are also all clearly contemplated as within the scope of the present invention.

All processes that are controlled by this novel signal transduction pathway, involving plasmodesmal macromolecular transport, can be modified to alter plant function. Furthermore, novel molecules can be engineered to utilize this cell-to-cell and phloem long-distance transport route. Such a strategy will allow for the effective delivery of xenobiotic agents for control of a wide range of pests, as well as modified or genetically engineered plant proteins that will potentiate control over gene expression and cellular physiology.

In Example 1, the influence of the 30-kDa movement protein of tobacco mosaic virus (TMV-MP) on carbon partitioning in transgenic tobacco plants (*Nicotiana tabacum* L. cv Xanthi) expressing the TMV-MP was investigated. Using

reciprocal grafting of transgenic tobacco plants expressing this movement protein and vector control plants, as well as transgenic tobacco plants expressing the TMV-MP in phloem cells only, it was shown that the interactive site involved in carbon allocation to roots is localized to the mesophyll tissue. Biomass partitioning experiments conducted on transgenic plants in which various deletion mutant forms of the TMV-MP (two of which included deletions in the domain responsible for increasing the size exclusion limit) were expressed revealed that the TMV-MP exerts its influence over carbon allocation via a mechanism that is completely independent of the TMV-MP-induced increase in plasmodesmal size exclusion limit. Furthermore, small N-and C-terminal deletions in the MP revealed the complexity in the interactions likely involved between the MP and an endogenous regulatory mechanism. The TMV-MP thus interferes with an endogenous signal transduction pathway that involves macromolecular trafficking through plasmodesmata to regulate biomass partitioning between the source and various sink tissues.

In Example 2, transgenic tobacco plants (*Nicotiana tabacum* L. cv. Xanthi) expressing wild-type or mutant forms of the 30-kDa movement protein of tobacco mosaic virus (TMV-MP) were employed to study the effects of the TMV-MP on carbon metabolism in source leaves. Fully expanded source leaves of transgenic plants expressing the TMV-MP were found to retain more newly fixed ^{14}C compared with control plants. Analysis of ^{14}C-export from young leaves of TMV-MP plants, where the MP is yet to influence plasmodesmal size exclusion limit, indicated a similar pattern, in that daytime ^{14}C export was slower in TMV-MP plants as compared to equivalent-aged leaves on control plants. Pulse-chase experiments were used to monitor radioactivity present in the different carbohydrate fractions, at specified intervals following $^{14}CO_2$ labeling. These studies established that the TMV-MP can cause a significant adjustment in short-term ^{14}C-photosynthate storage and export. That these effects of the

TMV-MP on carbon metabolism and phloem function were not attributable to the effect of this protein on plasmodesmal size exclusion limits, per se, was established using transgenic tobacco plants expressing temperature-sensitive and C-terminal deletion mutant forms of the TMV-MP. Collectively, these studies establish the pleiotropic nature of the TMV-MP in transgenic tobacco. The results suggest potential sites of interaction between the TMV-MP and endogenous processes involved in regulating carbon metabolism and export.

As reported in Example 3, coinjection of KN1 and fluorescently labelled *Knotted* mRNA resulted in the efficient transport of mRNA from the target cell into the cells of the surrounding tissues. As expected, injection of fluorescently labelled *Knotted* mRNA alone resulted in the confinement of the fluorescent probe to the injected cell. Further, coinjection of the nonfunctional KN1 M11Y51 mutant and fluorescently labelled *Knotted* mRNA also resulted in the mRNA being confined to the target cell. This clearly demonstrates that a plant-encoded protein engages in its own cell-to-cell transport, as well as the transport of its mRNA.

In Example 4, it is shown that TMV-MP alters plant growth response to light intensity. When vector control, wild type and deletion mutant forms of TMV-MP transgenic plants were grown under high light conditions, the wild type showed slight reductions in height and weight compared with the control. But, the deletion mutant exhibited an extremely different phenotype from the other two: plant height and total dry weight were greatly reduced. However, mean internodal length was not affected, and the root-to-shoot ratio of the deletion mutant was similar to that of the wild type. Then, under low light conditions, all three plant lines showed reduced root-to-shoot ratios, but the difference between transgenic and control plants seen under high-light conditions was not carried through; values for all three lines were the same. Yet, regarding internodal length and plant height, control plants increased in both; and, the wild

type increased in the former, but not in the latter. The deletion mutant showed no response to low light.

In Example 5, an analysis of movement of the protein and RNA encoded by the maize *knotted1* (*kn1*) homeobox gene (8) is reported. *In situ* and immunolocalization experiments show that the protein product, KN1, moves between adjacent cell layers. Microinjection studies in maize and tobacco show similar results. And, comparison of wild type with various alanine scanning mutants of KN1 showed the mutants to have a reduced capacity to dilate plasmodesmata and potentiate their own cell-to-cell transport.

The present invention has immediate practical value in more precise characterization of the molecular steps involved in the process of macromolecular transport. This will then potentiate efficient manipulation of resource allocation without the complications associated with traditional genetic approaches.

The invention may be better understood with reference to the accompanying examples, which are intended for purposes of illustration only and should not be construed as in any sense limiting the scope of the present invention as defined in the claims appended hereto.

EXAMPLES

Example 1

Transgenic *Nicotiana tabacum* L. cv Xanthi expressing the TMV-MP and various deletion mutant forms of the MP were obtained from Dr. Roger Beachy, Scripps Institute, La Jolla, California.

Transgenic line 277 expressed the TMV-MP, while line 306 was a control which contained only the plasmid vector [Deom et al. 1987, *supra*]. Details relating to the different mutant forms of the TMV-MP utilized in this example are provided in Table 1. Transgenic lines 277 and 306 were used in the graft experiments. Most plant lines were R_5 to R_8 and were homozygous for the MP construct. In addition, it was already confirmed that the effects of the TMV-MP on carbon

metabolism and biomass partitioning were not due to somaclonal variation or position effects associated with insertion sites [Lucas et al. 1993, *supra*]. The results obtained on the independent transformed tobacco plants used in the present study, which involved a wide range of TMV-MP mutants (see Table 1), further confirmed this finding.

Seeds were germinated on a soil mix, and after four weeks, seedlings were transplanted into 17.5 cm diameter plastic pots containing Yolo sandy loam. Plants were watered with 1/2X Hoagland's solution [Hoagland D. R. & Arnold D. I. (1938) The water-culture method for growing plants without soil. *California Agricultural Experimental Student Circular* **357,** 1-39] twice daily. Five to six weeks after transplanting (70-75 days post germination), plants were separated into leaves, stems and roots and dried at 70°C for 2 days. Dry weights were used to calculate tissue biomass and to compute root-to-shoot ratios. Routine measurements of photosynthetic rates were made during all growth experiments using a portable infra-red gas-exchange system (model LI-6200; LiCor, Lincoln, Nebraska). A fully developed leaf (leaf # 5, leaf # 1 being the youngest leaf to reach 5 cm at the time of measurement) was enclosed in a 1-l chamber such that a 12-cm^{-2} area was illuminated (Schott illuminator, KL-1500, Walz, Germany; approx. 1000 μmol m^{-2} s^{-1}). The CO_2 concentration in the chamber varied between 350-370 ppm and relative humidity ranged between 30-45%. CO_2 uptake was measured and using the above parameters, rates of CO_2 exchange were computed.

Plants used in experiments reported in Tables 3 and 4 were grown in a greenhouse under autumn conditions (September-November; 10 hours natural sun light). Light intensities at mid-day ranged between 1200 and 1500 μmol m^{-2} s^{-1}, whereas during the early morning and at dusk, the light intensity was about 400 μmol m^{-2} s^{-1}. To extend the photoperiod, these plants were supplemented with light from high intensity metal halide lamps (600-700 μmol m^{-2} s^{-1}) during the last 4 hours of a 14 hour day. Day/night

temperatures were 26°C±3.0/18°C±4.0 , respectively. These biomass partitioning experiments were repeated on plants grown under summer conditions (May-August; environmental conditions as for Tables 2 and 6). In graft, TMV-infection and C-terminal deletion (10 amino acids) (Tables 2, 6, and 7), plants were grown under the longer photoperiods of mid-summer (16 hours). Light intensity at mid-day was 1200-1500 μmol m^{-2} s^{-1}, while in the morning and evening values ranged between 700-950 μmol m^{-2} s^{-1}. Further, these plants were subjected to 30°C± 5.0 /22°C± 2.0 day/night temperature regimes, conditions that were directly comparable to those previously employed [Lucas et al. 1993, *supra*]. In all experiments, the location of a particular plant was selected at random in order to compensate for any minor variation in microclimate within the greenhouse.

Stocks and scions of MP-expressing transgenic tobacco line 277 and vector control line 306 were grafted, reciprocally. A "v" shaped notch was cut in the stem of the stock plant after removing the shoot at the second or third internode above the soil line. The lower remaining leaves on the stock were removed and the scion stem base was cut to a wedge and then inserted into the notch made in the stock. Both scion and stock were held together at the graft region using tygon tubing. The lower mature leaves on the scion were removed to reduce transpiration. The grafted plants were maintained in a mist chamber for two to three weeks until the graft had fused. Day/night temperatures in the mist chamber were 25°C/18°C, respectively. Grafted plants were then transferred to the greenhouse where they were grown for a further three weeks before being harvested for dry matter partitioning analysis.

For detection of TMV-MP in plants expressing the *TMV-MP* gene under phloem specific *rolC* promoter, leaf tissues of transgenic plant lines RMN-1, RMn-1, 277 and of vector control line 306 were extracted in lithium-phosphate buffer (50 mM) containing iodo-acetic acid (2.0 mM) and 2-mercaptoethanol (120 mM, pH 7.2) with a ratio of 1 g of

tissue to 7 ml of buffer. Levels of TMV-MP in all other lines employed in the present study were well established in earlier reports [Deom et al. 1987, *supra*; Wolf et al. 1989, *supra*; Wolf S., Deom C. M., Beachy R. N. & Lucas W. J. (1991) Plasmodesmatal function is probed using transgenic tobacco plants that express a virus movement protein. *Plant Cell* **3**, 593-604; Berna A., Gafny R., Wolf S., Lucas W. J., Holt C. A. & Beachy R. N. (1991) The TMV movement protein: Role of the C-terminal 73 amino acids in subcellular localization and function. *Virology* **182**, 682-689; Gafny R., Lapidot M., Berna A., Holt C. A., Deom C. M. & Beachy R. N. (1992) Effects of terminal deletion mutations on function of the movement protein of tobacco mosaic virus. *Virology* **187**, 499-507; Lapidot M., Gafny R., Ding B., Wolf S., Lucas W. J. & Beachy R. N. (1993) A dysfunctional movement protein of tobacco mosaic virus that partially modifies the plasmodesmata and limits virus spread in transgenic plants. *The Plant Journal* **4**, 959-970]. The leaf homogenate was centrifuged at 10,000g for 10 minutes, and soluble (cytoplasm) as well as insoluble (cell wall and membranes) fractions were separated. Proteins in both of these fractions were separated by one-dimensional polyacrylamide gel-electrophoresis. Electrophoretic blotting procedures were followed as described [Towbin H., Staehelin T. & Gordon J. (1979) Electrophoretic transfer of proteins from polyacrlamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354] with some modifications. Proteins were transferred onto Immobilon membrane (Millipore), which was then blocked in bovine serum albumin for one hour. The membrane was then incubated in phosphate-saline buffer containing antibody raised against the TMV-MP. The cross-reaction between the 30-kDa TMV-MP and the antibody against the TMV-MP was observed by color reaction using horse radish peroxidase as the secondary antibody.

Tobacco plants (lines 306 and 277) having six fully expanded leaves (mid-summer-grown plants) were inoculated on

the 4th leaf (counting from the top) with 0.5 mg ml^{-1} TMV in phosphate buffer (pH 7.2) using Carborundum (400 mesh). Plants were transferred to shade conditions (light intensity approx. 150 μmol m^{-2} s^{-1}) for two days before being returned to normal full sunlight conditions in the greenhouse (see above for growth conditions). Symptom development was recorded and at the time of harvesting the plants had 12-14 systemically infected leaves, showing normal chlorotic-mosaic symptoms associated with TMV infection.

To ascertain whether the TMV-MP is required, in both shoots and roots, to exert its influence over biomass partitioning, a series of graft experiments were conducted. Shoots of transgenic line 277, in which the TMV-MP was being expressed, were grafted on vector control line 306, and vice versa. As illustrated in Table 2, when the scion (shoot) was 277 in nature, the root-to-shoot ratio was low, whereas when the scion was from line 306, the root-to-shoot ratio was relatively high and comparable to values obtained on grafted plants in which both scion and stock were derived from line 306 (Table 2). These results established that the TMV-MP was only required in source leaf tissues in order for it to alter the root-to-shoot ratio.

To further refine the site(s) of interaction between the TMV-MP and the putative endogenous control system that regulates carbon partitioning, transgenic plants were employed in which expression of the TMV-MP was confined to cells of the phloem (vasculature). The presence of the TMV-MP in these plant lines (RMn-1 and RMN-1) was confirmed by western blot analysis using antisera against the TMV-MP. As illustrated in the data presented in Table 3, when the TMV-MP was localized to the phloem, such plants were similar in phenotype to the vector control line (cf. lines RMn-1, 306 and 277). Note that although the leaf biomass was similar in lines 306, 277 and RMn-1, stem and root mass was significantly lower in line 277 compared with lines 306 and RMn-1. A second experiment was performed, in which Xanthi NN plants were used, and again, as shown by the data presented

in Table 3, expression of the TMV-MP within the phloem tissues had no detectable influence over plant weight, leaf, stem and root mass, or carbon partitioning, as reflected by the root-to-shoot ratio. These data suggest that the TMV-MP had only to be present in the mesophyll tissue in order to exert its influence over carbon allocation.

Earlier studies established that the influence of the TMV-MP, in terms of increasing the SEL of secondary plasmodesmata, was also restricted to the mesophyll and mesophyll/bundle-sheath boundaries [Ding et al. 1992, *supra*]. To explore whether a direct relationship existed between the ability of the TMV-MP to increase plasmodesmal SEL and to alter biomass partitioning, experiments were performed on a range of MP deletion mutants that spanned the domain in the TMV-MP (amino acids 195-213) responsible for the dilation of secondary plasmodesmata [Berna et al. 1991, *supra*]. Since these mutant forms of the TMV-MP were expressed in tobacco genotypes nn and NN (isogenic, or near isogenic lines), non-transformed tobacco plants were first analyzed, and it was found that biomass partitioning and root-to-shoot ratios were comparable in both genotypes. It was next established that both genetic lines yielded statistically identical biomass partitioning results in terms of non-transformed and vector control transformed plants.

The first two TMV-MP deletion mutants used in this series of experiments (lines MN-1 and MN-2) had the C-terminal 33 and 55 amino acids deleted, respectively, but they still retained the ability to dilate plasmodesmata to the level detected in TMV-MP transgenic plants [Berna et al. 1991, *supra*]. As shown in Table 4, transgenic tobacco line MN-1 showed a slight reduction in its ability to override the endogenous control over carbon partitioning, when compared with that of the full-length TMV-MP. In transgenic tobacco line MN-2, a minor alteration in the ability of the mutant TMV-MP to reduce carbon allocation to the lower stem and roots was also observed. A deletion within the SEL domain of the TMV-MP (line Mn-3) had no significant effect on growth or

root-to-shoot ratios, when compared with values obtained on line 277. Furthermore, a similar result was obtained when the entire SEL domain was removed (line Mn-4; see Table 4). Note that in mutant lines Mn-3 and Mn-4 the plasmodesmal SEL was identical to line 306, being approx. 900 Da.

To further explore the relationship between SEL and alteration in carbon allocation, biomass partitioning experiments were performed on a mutant form of the TMV-MP in which amino acids 3-5 had been deleted from the N-terminus [Lapidot et al. 1993, *supra*]. Transgenic tobacco plants expressing this mutant (line Mn-5) exhibited a reduced SEL of 3.9 kDa compared with 9.4 kDa in plant line 277 [Lapidot et al. 1993, *supra*]. Interestingly, these plants showed an even more drastic phenotype, with leaf, stem and root mass being reduced to 50% of the values measured in plant line 277. This reduction in plant biomass was not due to an effect of the mutant TMV-MP on photosynthesis, as under the growth conditions used in the present study, transgenic plants expressing all forms of the TMV-MP had measured photosynthetic rates that were comparable to the rates measured in control lines; i.e., 11.6 μmol CO_2 m^{-2} s^{-1}. It is interesting to note that even though Mn-5 had an overall reduction in biomass partitioning, root-to-shoot ratios were comparable to those measured in 277 and other lines expressing mutant forms of the TMV-MP. Transgenic line Mn-6 represented an ideal internal control for the latter experiment, as this line did not contain detectable levels of TMV-MP, although the gene was present in these plants [Lapidot et al. 1993, *supra*]. These plants showed similar characteristics of biomass partitioning and root-to-shoot ratios when compared with vector control line 306 (see Table 4).

As part of the growth analysis, performed on the transgenic tobacco plants expressing the various mutant forms of the TMV-MP, plant height and number of leaves were measured at the time of harvest. The data presented in Table 5 are consistent with the conclusions drawn from biomass data

presented in Table 4. Interestingly, mutant line MN-2 again showed signs of reverting towards the phenotype of vector control plants. However, in all other respects these data indicated that the influence of the TMV-MP on plant growth (as reflected by plant height and leaf number) was independent of the TMV-MP-induced change in plasmodesmal SEL. Note again that whereas Mn-5 represented the smallest phenotype, with the fewest leaves, plant line Mn-6 was identical to the vector control line 306. Collectively, these results established that the TMV-MP-induced alteration in carbon allocation was not associated with the ability of the TMV-MP to increase plasmodesmal SEL.

Over the course of these experiments, plants were grown under both summer (May-August) and autumn (September-November) conditions. While each repeat experiment confirmed the explicit influence of the TMV-MP on biomass partitioning and root-to-shoot ratio, the absolute values displayed a trend in which all values were larger in summer grown plants.

In view of the established significant effects that the TMV-MP has when constitutively expressed in transgenic tobacco plants, the effect of the TMV-MP produced during normal plant infection by TMV was explored. As illustrated in Table 6, TMV infected 306 plants had reduced root biomass and their subsequent root-to-shoot ratio was also lowered from the uninfected control value of 0.11 to 0.07 (i.e., a value identical to that measured on the uninfected 277 plant line). Interestingly, TMV infection of plant line 277 failed to further reduce root mass or root-to-shoot ratio. These results establish that the TMV-MP produced in the course of infection results in an identical effect on biomass partitioning as that observed when the TMV-MP is constitutively expressed in transgenic plants. Since TMV infection of 277 plants failed to further influence biomass partitioning, in the presence of TMV-MP expressed within these plants, it would appear that the level of the MP in plant line 277 is already at a sufficient level to exert its

full influence over the TMV-MP induced alteration in carbon allocation to the roots.

The effect of a small, C-terminal 10 amino acid, deletion in the TMV-MP on sugar metabolism was investigated. Transgenic plants expressing this mutant form of the MP exhibited a normal pattern of sugar metabolism with sucrose, glucose, fructose, and starch levels all reflecting diurnal patterns equivalent to control plant line 306. In view of this important finding a parallel study was performed to determine the effect of this mutant TMV-MP on biomass partitioning. As illustrated in Table 7, two independently transformed lines (Mn-1 and Mn-2) exhibited statistically identical biomass partitioning characteristics. Note that in this experimental series, plant line 277 had a root-to-shoot ratio of 0.07 whereas both plant lines Mn-1 and Mn-2 had root-to-shoot ratios of 0.10 to 0.12, which were statistically identical to the value of 0.11 measured on plant line 306. Western blot analysis indicated that the level of TMV-MP in Mn-1 and Mn-2 was approximately 25% of that in line 277. This level is consistent with previous reports [Berna et al. 1991, *supra*]. These data establish that a C-terminal 10 amino acid deletion in the MP abolished its ability to alter both biomass partitioning and carbon export/sugar metabolism (Table 7). A comparison of growth characteristics between plant lines 306, Mn-1, Mn-2 and 277 also supported this conclusion in that the first three had identical characteristics, while 277 plants showed the same differences as previously illustrated in Table 5.

Based on the grafting experiments (see Table 2) and studies performed on transgenic plants in which expression of the TMV-MP was confined to the phloem (see Table 3), the presence of the TMV-MP within the mesophyll tissue is sufficient for it to exert its influence over carbon allocation and biomass partitioning within the plant. The observation that normal root-to-shoot ratios (equivalent to vector control RGN-1, 3001 and 306) were present in plants in which the TMV-MP was restricted to the phloem is consistent

with previous findings establishing that the TMV-MP is ineffective within the phloem, in that it is unable to increase plasmodesmal SEL between the cells of the phloem [Ding et al. 1992, *supra*]. These results support the hypothesis that the TMV-MP is required, specifically, in mesophyll tissue in order to exert its effects over biomass partitioning.

Recent studies provided direct experimental proof that viral MPs have the capacity to effect their trafficking through mesophyll plasmodesmata [Fujiwara T., Giesman-Cookmeyer D., Ding B., Lommel S. A. & Lucas W. J. (1993) Cell-to-cell trafficking of macromolecules through plasmodesmata, potentiated by the red clover necrotic mosaic virus movement protein. *Plant Cell* **5**, 1783-1794; Noueiry A. O., Lucas W. J. & Gilbertson R. L. (1994) Two proteins of a plant DNA virus coordinate nuclear and plasmodesmal transport. *Cell* **76**, 925-932; Waigmann et al. 1994, *supra*]. In view of this property, the possibility exists that movement proteins synthesized within the leaf may undergo extensive trafficking into all of the surrounding tissues. Clearly, if the TMV-MP could traffic from vascular into mesophyll tissue, a change should have been observed in root-to-shoot ratio in plant line RMn-1 and RMN-1. The absence of such an effect is consistent with the observation that macromolecular trafficking through plasmodesmata is always correlated with an increase in SEL to 9.4 kDa or above. The inability of the TMV-MP to interact with phloem plasmodesmata to cause such an increase in SEL [Ding et al. 1992, *supra*] implies that the protein would not be able to traffic within this tissue, nor would it be capable of exiting across the bundle-sheath plasmodesmata to enter the mesophyll. The trafficking of TMV-MP, located in the mesophyll, would similarly be restricted to plasmodesmal transport within this tissue, but also would include transport into the bundle-sheath cells [Ding et al. 1992, *supra*]. Thus, the mechanism by which the TMV-MP elicits a change in biomass partitioning most likely involves a

perturbation to an endogenous control mechanism that originates from within the mesophyll.

The experiments performed on transgenic plants expressing mutant forms of the TMV-MP show that the TMV-MP-induced alteration in carbon partitioning is independent of the effect of the ability of this MP to interact with and increase the SEL of mesophyll secondary plasmodesmata. C-terminal deletion mutations both inside and at the borders of the MP domain associated with increasing the plasmodesmal SEL [Lucas W. J. & Gilbertson R. L. (1994) Plasmodesmata in relation to viral movement within leaf tissues. *Annual Review of Phytopathology* **32,** 387-411] had little or no effect on carbon partitioning, as reflected by biomass partitioning and root-to-shoot ratios (see Table 4). Indeed, removal of both the C-terminal 73 and 116 amino acids from the TMV-MP (line Mn-3 and Mn-4) prevented these mutant MPs from entering the plasmodesmata, with most of the protein residing in the soluble (cytoplasmic) fraction [Berna et al. 1991, *supra*]. Yet, transgenic plants expressing these mutant forms of the TMV-MP had root-to-shoot ratios equivalent to plant line 277.

The most pronounced effect on the phenotype of transgenic tobacco plants expressing the TMV-MP was observed in plants expressing a mutant form in which the N-terminal 3-5 amino acids were deleted. These plants exhibited an overall reduction in plant morphology in comparison to control line 306 (see Table 5). This effect is quite remarkable given that the level of MP in the mesophyll tissue of plant line Mn-5 was approximately 1/20th of that measured in plant line 277. Additionally, Mn-5 was dysfunctional, in that although it entered the plasmodesmata, the levels were extremely low (below the detectable limits of immuno-cytochemistry) [Lapidot et al. 1993, *supra*], and it was incapable of increasing the SEL beyond 3.9 kDa. Again, the influence of this movement protein on biomass partitioning is inconsistent with a simple increase in plasmodesmatal SEL.

The influence of Mn-5, on the overall phenotype of the plant, suggests that the N-terminus of the TMV-MP may contain a domain which interacts with an endogenous regulatory mechanism involved in carbon partitioning. This would imply that the C-terminal portion of the MP serves to mediate in viral systemic infection. This conclusion is supported by the recent finding that the N-terminal 1-126 amino acids are completely dispensable in terms of TMV-MP trafficking through mesophyll plasmodesmata [Waigmann et al. 1994, *supra*]. Furthermore, transgenic plants expressing the MP of cucumber mosaic virus (CMV-MP), which has a C-terminus that is similar to that of TMV-MP, had no effect on plant phenotype or root-to-shoot ratio.

As the level of the TMV-MP in plant lines Mn-1 and Mn-2 was lower (approx. 25%) than that detected in line 277, the possibility must be considered that this level is insufficient to effect any change in biomass partitioning. Since the TMV-MP in lines Mn-1 and Mn-2 caused "normal" TMV-MP-induced increase in plasmodesmal SEL and complemented infection by a TMV (-MP) construct [Berna et al. 1991, *supra*], it would appear that adequate MP is present within the mesophyll tissue. This conclusion is also supported by the fact that plant line Mn-3, which had a comparable level of TMV-MP to plant lines Mn-1 and Mn-2 [Berna et al. 1991, *supra*], exhibited root-to-shoot ratios equivalent to those observed in plant line 277 (see Table 4). In addition, plant line Mn-5, which had MP levels of approx. 1/20 of those present in plant line 277, exhibited biomass partitioning equivalent to plant line 277 (Table 4). Finally, parallel sugar and starch analysis, performed on these deletion mutant plant lines, revealed equivalent levels within plant lines Mn-3, Mn-4 and 277.

The important point established by studies on plant lines Mn-1 and Mn-2 is that a common site of action, in the endogenous regulatory mechanism, may be involved in mediating the TMV-MP-induced alteration in both sugar metabolism and biomass partitioning. Alternatively, the influence of the

TMV-MP could reflect a direct effect on one physiological process with a secondary effect on the other. The complexity of these regulatory signals involved in the orchestration of carbon metabolism and partitioning was also illustrated by the finding that sink strength could override the effect of the TMV-MP on net carbon export, but did not negate the influence of the MP on sugar accumulation in mature source leaf.

The present results establish that the TMV-MP exerts its effect on carbon allocation and biomass partitioning from a site located within the mesophyll tissue. Further, the mode of action of the TMV-MP, in terms of altering biomass partitioning and root-to-shoot ratio, appears to be completely independent of the mechanism by which this MP acts to increase the SEL of mesophyll secondary plasmodesmata. Finally, source leaves of TMV-MP transgenic tobacco plants have elevated levels of sugars and starch, although their photosynthetic rates are equivalent to control lines, suggesting that either the transport of sugars out of the leaf is reduced, or that photosynthate metabolism (sugars and starch) is affected.

Several possible mechanisms can be postulated to account for the way in which the TMV-MP alters carbon allocation and biomass partitioning. One possible site for these effects might be located at the plasmodesmata where the MP may interfere with sucrose movement from the mesophyll into the phloem. However, there are presently no data consistent with active, vectorial, transport of sucrose (or any other small metabolite) through plasmodesmata. Nor is information available on whether sucrose interacts, directly, with any plasmodesmal protein(s).

The TMV-MP may exert its effects directly, or indirectly, by altering metabolism and/or membrane transport of sugars (glucose, fructose, sucrose, etc). Likely sites may reside at the chloroplast envelope, the tonoplast (mesophyll) or the plasma membrane (mesophyll and/or phloem), and involve an interaction between the TMV-MP and either the

sugar transporter or regulatory elements that may control carbon allocation. Such an interaction may possibly explain the observed changes in glucose and fructose compartmentation, as well as starch metabolism, in TMV-MP transgenic tobacco plants. However, it is difficult to reconcile the MP-induced changes in sucrose levels on the basis of this model, as the major site of influence of the TMV-MP would have to be at the companion cell-sieve element complex where sucrose loading is thought to take place. This site of action is inconsistent with the experimental data reported herein, in that plants in which TMV-MP synthesis is restricted to the vascular tissue exhibit carbon allocation and biomass partitioning patterns that are identical to control plants (Table 3). This suggests that a more complex interaction may be involved in the regulation of photosynthate movement from the mesophyll to the companion cell-sieve element complex. Furthermore, the finding that the presence of the TMV-MP in leaf tissues (within the mesophyll) results in a significant reduction in biomass being partitioned to the lower stem and root tissues (Tables 2, 4 & 7) also implies the involvement of a complex regulatory mechanism for the control of photosynthate utilization.

In light of findings that plasmodesmata are capable of transporting endogenous proteins [Fisher D.B., Wu Y. & Ku M.S.B. (1992) Turnover of soluble proteins in the wheat sieve tube. *Plant Physiology* **100**, 1433-1441] as well as viral proteins [Lucas & Gilbertson 1994, *supra*], a model based on this established macromolecular (protein, RNA/DNA) trafficking capability of higher plant plasmodesmata is proposed, wherein the companion cell-sieve element complex within the source leaf would function as the control center in terms of establishing priorities with respect to carbon delivery to various sinks throughout the plant. Further, photosynthesis and short-term carbohydrate storage, within the mesophyll, would be regulated by output signals (macromolecular) that traffic via plasmodesmata from the

companion cell to the mesophyll, where they function by interacting with either sugar transport systems (tonoplast or chloroplast envelope) or key enzymes in the metabolic pathway leading to sucrose synthesis [Lucas W. J. & Wolf S. (1993) Plasmodesmata: the intercellular organelles of green plants. *Trends in Cell Biology* **3**, 308-315]. The return loop of this signal transduction cycle would involve input signals from the mesophyll that are transported via plasmodesmata to the companion cell-sieve element complex. These input signals would act to "inform" the companion cell-sieve element complex of the status of photosynthesis within the mesophyll (i.e., current rate of net carbon fixation under prevailing conditions).

In the framework of this macromolecular trafficking signaling model, the TMV-MP would act as a competitive analogue to an endogenous protein that functions as an essential component of the input signal(s) that traffics, via plasmodesmata, from the mesophyll to the companion cell-sieve element complex. These two protein homologue (the TMV-MP and the endogenous input signaling protein) would compete for a common plasmodesmal binding site, with the TMV-MP reducing the transport of the endogenous input signal. A reduction in the level of this input signal (protein) is proposed to result in a change in protein synthesis (gene expression) within the companion cell, resulting in an alteration in both the rate at which sucrose is loaded into the phloem and the set-point [Mooney & Winner 1991, *supra*] in carbon delivery to the roots. The change in set-point for carbon delivery would involve a modulation in trafficking of proteins from the companion cell to the sieve element. The immediate result of the change in phloem loading would be an increase in sucrose levels (on a diurnal basis) within the mesophyll [Lucas et al. 1993, *supra*]. The alteration in the set-point of biomass partitioning, mediated by a change in the delivery of informational macromolecules to the root (via the phloem), would give rise to plants having a significant reduction in root-to-shoot ratios (Tables 3, 4 & 6).

The experimental results presented in Tables 4 and 5 provide support for the claim that control can be exerted over plant development through an interaction with plasmodesmal macromolecular trafficking. In this case, transgenic plants expressing mutant forms of the TMV-MP exhibited an overall reduction in plant growth. This was the direct result of expressing a mutant form of the TMV-MP in which amino acids 3,4 & 5 had been deleted (mutant Mn-5 in Tables 4 & 5). In these transgenic tobacco plants, the presence of a dysfunctional Mn-5 TMV-MP gave rise to a down-regulation of overall plant growth. Whereas leaf number was little affected, total plant weight and plant height were reduced to 41% and 55%, respectively, of control plants (Tables 4 & 5). A comparison between these results and those obtained with plants expressing a C-terminal 10 amino acid deletion mutant of the TMV-MP (see Table 7), establish that the effects on plant growth must be directly attributable to the presence of the mutant form of the TMV-MP.

These results establish that expression of a dysfunctional form of a protein that has the molecular properties to enable it to interact with and/or traffic cell-to-cell through plasmodesmata can be used to control overall plant size. Manipulation of such dwarf characteristics should have general application in horticultural practices.

Example 2

Transgenic tobacco plants (*Nicotiana tabacum* L. cv. Xanthi) expressing wild-type (plant lines 277 [Deom et al. 1987, *supra*] and 2004 [Deom et al. 1991, *supra*], temperature-sensitive mutant (ts, MPP 154A) line 2-72 [Wolf et al. 1991, *supra*] and C-terminal (10 amino acids) deletion mutant line MP1 [Berna et al. 1991, *supra*] forms of TMV-MP, as well as vector control tobacco plants (lines 306, 3001; transformed but without the *MP* gene), were used in the present experiments. Three-week-old seedlings were transferred into plastic pots (15 cm diameter) and plants were grown in an insect-free, temperature-controlled greenhouse (approx. 25°C

day/18°C night). In some experiments, the temperature within this same greenhouse was raised to 34°C/32°C (day/night, respectively). Plants were grown under natural sunlight having a midday average photon flux density of 1500 μmol.m^{-2}.s^{-1}.

Net photosynthesis (measured as CO_2 uptake) was determined using a closed-portable infrared gas exchanged system (LI-6200, LICOR Inc., Lincoln, Nebraska, USA). An attached leaf was placed in a 11 lexan chamber such that a 10 cm^2 area was exposed to light and gas flow. The youngest fully expanded leaf (#5 or #6, with leaf # 1 being the last leaf to achieve a length of 5.0 cm) was used in these experiments. All measurements (photosynthesis and respiration) were carried out on well-watered plants during the late morning h (10-12 noon) on bright, sunny days. The initial CO_2 concentration in the chamber was 340±10 μl l^{-1}, and a 30 s measurement was started immediately after a reduction in CO_2 concentration was detected. Dark respiration was measured by covering the leaf chamber with a black cloth and measurements were started immediately after an increase in CO_2 concentration was observed. Negative values of photosynthesis were interpreted as dark respiration.

Carbohydrate content within leaves was determined as previously described [Lucas et al. 1993, *supra*]. In brief, soluble sugars were extracted in 80% ethanol from leaf discs (1.5 cm^2). After evaporating the supernatant to dryness, sugars were redissolved in H_2O and then filtered through a 0.45 μm membrane (Whatman, Clifton, New Jersey, USA). Sugar separation was carried out on an analytical HPLC system (LDC Anal., Reviera Beach, Florida, USA), fitted with a Sugar-Pak I column (6.5 mm · 300 mm; Waters Associates, Milford, Mass., USA) using an LDC refractive-index detector (Refractor Monitor IV). Starch content was determined on the ethanol-water extracted leaf discs following starch conversion by amyloglucosidase (Cat. No. A-7255; Sigma Chemical Co., St. Louis, Missouri, USA). Starch content, as glucose

equivalents, was measured using the Sigma (HK) quantitative glucose determination kit.

Tobacco leaves were labeled with $^{14}CO_2$ by using a pulse-labeling system. Experiments were carried out in the greenhouse, during the late morning h on bright days, under optimal photosynthetic conditions. An attached tobacco leaf was sealed into a 4l Plexiglas chamber where it was held between two layers of nylon monofilament. A volume of 60 cm^3 of $^{14}CO_2$ was then released into the chamber (40 s) to give an initial specific activity of $2 \cdot 10^5$ $Bq \cdot mg^{-1}$ carbon. It took less than 20 min for the CO_2 concentration in this chamber to be reduced to the compensation point, by which time the $^{14}CO_2$ would have been assimilated into photosynthetic products. At this point, the leaf was released from the chamber and was used for analysis of ^{14}C-photosynthate export.

Rate of reduction in radioactivity of either total leaf ^{14}C or ^{14}C-labeled carbohydrates was determined from time course measurements that commenced immediately after a leaf was released from the $^{14}CO_2$-labeling chamber. Total radioactive content within each ^{14}C-labeled leaf was determined using one of the following two methods. First, at various time intervals, leaf discs were punched from an attached leaf and were then dissolved in tissue solubilizer (Soluen-350; Packard Instrument Co., Inc., Downers Grove, Illinois, USA). After 3-5 d the radioactivity was measured on a Betamatic liquid scintillation counter (Kontron Instrument, Zürich, Switzerland) using Ultima-gold scintillation cocktail (Packard Instrument Co.). Second, a portable, Geiger-Müller tube (RAM-DA, Rotem Industries, Be'er Sheva, Israel) containing a circled ß counter, (Model GM-10, diam. 4 cm) was placed on the adaxial surface, to the side of the main vein, in the mid-region of an intact ^{14}C-labeled leaf; data were collected (100 s sampling period) at appropriate times over a 24 h experimental period. A comparison between *in planta* ß-radiation detection (Geiger-Müller) and scintillation-based ^{14}C analysis demonstrated that the two methods yielded data that were

highly correlated over the range of radioactivity levels employed in the present experiments. Radioactivity within an intact leaf was assayed using the portable ß counter system following $^{14}CO_2$ feeding. Then, three leaf discs were punched from the detection site and dissolved for 3-5 days in Soluene-350. Radioactivity within these discs was then assayed by scintillation spectrometry. In view of this finding, the portable β-radiation detector was used to perform non-destructive time course experiments on $^{14}CO_2$ pulse-labeled source leaves of TMV-MP transgenic (lines 277, 2004 and 2-72) and control (lines 306 and 3001) tobacco plants.

Partitioning of newly fixed carbon within leaf carbohydrates was determined in a manner that gave data on both concentration and radioactivity of each component. Sugars (sucrose, glucose and fructose) were identified and fractionated by HPLC. Radioactivity that eluted at the sample-front peak was collected separately and termed the liquid residue. Radioactivity in the starch fraction was measured after enzymatic conversion to glucose residues. Following carbohydrate extraction, the remaining leaf tissue was solubilized (Soluen-350) for 3-5 d prior to assaying for radioactivity (this fraction was termed the solid residue).

It has been established that TMV-MP transgenic plants (line 277) accumulated much higher amounts of carbohydrate during the day as compared to control tobacco plants (line 306); however, over the dark period, the level of all carbohydrates appeared to decline more rapidly in 277 than in 306 plants, often resulting in the establishment of comparable levels in both lines by the next morning [Lucas et al. 1993, *supra*]. The time course data presented in Fig. 1A indicate that the rate of reduction in radioactivity was significantly lower in *TMV-MP*-expressing tobacco leaves as compared to control plants. Plants were grown in a greenhouse (25°/18°C day/night) under natural sunlight with a midday average photon flux density of 1500 $\mu mol.m^{-2}.s^{-1}$. Radioactivity within intact leaves was assayed non-

destructively with a "Rotem" portable ß counter. Experiments were performed on fully expanded source leaves (#5 and 6) (**A**, **B**) and on younger, expanding source leaves (#2 and 3) (**C**). Plant lines 277 and 306 were used for experiments in **A** and **C**, while plant lines 2004 and 3001 were employed in the experiments presented in **B**. Values represent mean ± SE (n=5).

To further confirm the effect of the TMV-MP on the rate of reduction of ^{14}C-labeled photosynthate, a similar series of experiments on transgenic tobacco plants that expressed the TMV-MP in a slightly different genetic background (*N. tabacum* Xanthi NN) was performed. Plants homozygous for the *TMV-MP* gene and which had levels of TMV-MP within leaf tissues that were comparable to those measured in TMV infected plants (plant line 2004) [Deom et al 1991, *supra*], were chosen for these studies. As illustrated in Fig. 1B, plant line 2004 also exhibited a slower rate of reduction in the level of radioactivity that remained in the source leaves compared with equivalent leaves on the respective vector control plants (line 3001).

Pulse-labeling experiments, performed on young leaves (leaf #2) in which the TMV-MP would not yet have caused an increase in plasmodesmal SEL [Deom, C.M., Schubert, K.R., Wolf, S., Holt, C.A., Lucas, W.J., Beachy, R.N. (1990) Molecular characterization and biological function of the movement protein of tobacco mosaic virus in transgenic plants. *Proc. Natl. Acad. Sci. USA* **87**, 3284-3288; Ding et al. 1992, *supra*], demonstrated lower rates of reduction in the level of residual leaf radioactivity compared to fully expanded leaves (Fig. 1C). Although the rate of reduction of ^{14}C was similar between the two plant lines, line 277 consistently retained more ^{14}C photosynthate over a diurnal cycle (statistically significant at $P = 0.05$). These findings are in full agreement with an earlier study performed on a completely independent *TMV-MP* transformant (line 274) [Lucas et al. 1993, *supra*].

Analysis of ^{14}C-labeled and total photoassimilates

revealed a significant difference between 277 and 306 plant lines (Table 8). During the daylight hours the sucrose levels in TMV-MP transgenic plants were almost double those of control plants (Table 8). In control plants, ^{14}C-sucrose declined during the daylight hours to approximately 22% of the value measured shortly after the $^{14}CO_2$ labeling treatment; for plant line 277, this value decreased to only 50% over the same period. The decrease in ^{14}C-sucrose in line 277 represented the major portion of the total loss of radioactivity, whereas in control plants it represented about half of the total loss. The other fraction contributing to the total decline of radioactivity in control leaves (306) was the liquid residue (Table 8).

Interestingly, ^{14}C-glucose decreased by only 4% in line 277 compared with 30% in control plants, while in both lines ^{14}C-fructose increased during the period after $^{14}CO_2$ labeling, with a subsequent decrease being detected only in control plants. As expected, ^{14}C activity in the starch fraction remained relatively constant, in both lines, over the ensuing photoperiod. The solid residue fraction consisted of non-extractable metabolites and structural carbohydrates. Radioactivity in this component increased in both lines, probably due to turnover of cell wall components.

A temperature-sensitive (ts) TMV-MP, mutant MPP154A, was generated by changing the proline residue at amino acid 154 of the TMV-MP to alanine [Wolf et al. 1991, *supra*; Leonard, D.A., Zaitlin, M. (1982) A temperature-sensitive strain of tobacco mosaic virus defective in cell-to-cell movement generates an altered viral-coded protein. *Virology* **117**, 416-424; Ohno, T., Takamatsu, N., Meshi, T., Okada, Y., Nishiguchi, M., Kiko, Y. (1983) Single amino acid substitution in 30K protein of TMV defective in virus transport function. *Virology* **131**, 255-258; Deom et al. 1987, *supra*; Meshi, T., Watanabe, Y., Saito, T., Sugimoto, A., Maeda, T., Okada, Y. (1987) Function of the 30 kDa protein of tobacco mosaic virus: Involvement in cell-to-cell movement and dispensability for replication. *EMBO J.* **6**, 2557-2563].

Studies on plasmodesmal SEL in transgenic tobacco plants expressing this ts mutant TMV-MP (line 2-72) indicated that, under permissive temperatures (24°C; i.e. temperatures that permitted infection of these plants by a MP- strain of TMV), the SEL was elevated to levels identical to those measured in wild-type TMV-MP transgenic plants (line 277). However, under non-permissive temperatures (32°C), the SEL in plant line 2-72 was similar to that detected in control plants (line 306) [Wolf et al. 1991, *supra*]. Furthermore, this plant line was selected for the present study as homozygous plants were shown to express the ts-form of the TMV-MP to yield levels that were equivalent to those present in wild-type TMV-MP plants (lines 277, 274, etc.). Finally, previous studies on plant line 2-72 established that the ts TMV-MP did not undergo accelerated degradation under elevated temperatures [Wolf et al. 1991, *supra*].

Before examining the effects of temperature-induced alteration in TMV-MP-mediated increase in plasmodesmal SEL, it was necessary to ensure that temperature, per se, did not cause differential effects on photosynthesis or respiration in plant lines 277, 2-72 and 306. Photosynthesis and dark respiration measurements, performed on plants grown at 25°C daytime temperature, indicated that all plant lines examined yielded statistically identical parameters (Table 9). A similar situation was observed when plants were maintained under non-permissive temperatures (32-34°C, day and night), in that values for net photosynthesis and dark respiration were equivalent among plant lines 277, 2-72 and 306. However, the data presented in Table 9 show that the absolute rates of photosynthesis and dark respiration were considerably higher in the plants exposed to 32-34°C conditions. These control studies established that neither photosynthetic production nor local consumption differences could provide a basis to explain the MP-induced changes in carbon metabolism [Lucas et al. 1993, *supra*].

As shown in Fig. 2A, during the first hours after ^{14}C-labeling, the rate of reduction in radioactivity in source

leaves of plants maintained at 25°C was identical in transgenic plants expressing either the ts mutant or wild-type TMV-MP, but was slower compared to control plants (line 306). Plants were grown in the greenhouse under natural sunlight with a midday average photon flux density of 1500 $\mu mol.m^{-2}.s^{-1}$, under two temperature regimes of 25°/18°C day/night (left side) and 34°/32°C day/night (right side). Radioactivity was detected using a "Rotem" ß counter (Figs. 2A & 2B) and leaf discs were analyzed for starch (Figs. 2C & 2D), sucrose (Figs. 2E & 2F), glucose (Figs. 2G & 2H) and fructose (Figs. 2I & 2J). Five plants from each line and for each temperature regime were used in these experiments, and values represent mean ± SE. Interestingly, during the night, the rate of reduction in radioactivity within these source leaves was slower in the ts mutant TMV-MP transgenic plants compared to wild-type TMV-MP transgenic plants. Transgenic plants expressing either the ts mutant or the wild-type TMV-MP were also found to have similar sugar levels when the experiments were performed at 25°C (Figs. 2E, 2G & 2I). Consistent with previous findings [Lucas et al. 1993, *supra*], these sugar levels were always significantly higher in the TMV-MP plants compared with control plants. Interestingly, the starch levels in line 2-72 were intermediate between lines 277 and 306 (Fig. 2C).

Pretreatment of the three plant lines at non-permissive temperatures (32-34°C) for 72 h caused an increase in the overall rate of reduction of ^{14}C remaining in the source leaves of all plants tested (cf Fig. 2A & B). The interesting point to note in these experiments is that the rate of ^{14}C reduction in source leaves of plant lines 2-72 and 277 increased to such an extent that their values converged with those obtained on control plants (Fig. 2B). Under elevated temperatures, carbohydrate levels declined significantly in all plant lines, and as with ^{14}C-carbon export, the actual diurnal change in the levels of the individual sugars and starch was similar between the three plant lines (Fig. 2D, 2F, 2H & 2J). However, sucrose,

glucose and fructose levels still exhibited significant differences between lines 2-72, 277 and the control line 306.

A mutant form of the TMV-MP, in which the C-terminal 10 amino acids were deleted, retained full wild-type function, supporting viral infection and causing an increase in plasmodesmal SEL [Berna et al. 1991, *supra*; Gafny et al. 1992, *supra*]. Experiments performed on tobacco plants expressing this mutant form of the TMV-MP provided unequivocal support for the hypothesis that this viral protein functions in a pleiotropic manner in transgenic tobacco plants. As shown in Table 10, deletion of the terminal 10 amino acids appeared to eliminate the influence of the TMV-MP on carbohydrate metabolism within mature source leaves. Comparable levels of sucrose, glucose and fructose were detected in plant lines MP1 and 306, while, as expected, plant line 277 (expressing the wild-type *TMV-MP* gene) had elevated sugar levels. A similar situation was observed with respect to starch metabolism, with plant lines MP1 and 306 having near equivalent values for accumulation and hydrolysis over the experimental period (Table 10). It should be noted that, in plant line 277, the level of starch was higher than in either MP1 or 306 plants and that, in contrast to these plants, little hydrolysis occurred during the night (see also Fig. 2C where a similar situation is reported).

Again, to guard against the effects of positional insertion, somoclonal variation, etc., a second experiment was performed with a different, independent, transgenic tobacco line expressing a TMV-MP in which the C-terminal 10 amino acids were deleted (plant line MP1-1). For this experiment, the influence of the TMV-MP on sugar metabolism in plant line 2004 and 277 (compared with their respective control lines 3001 and 306) was also examined. As illustrated in Table 11, carbohydrate levels in transgenic tobacco plants expressing the C-terminal 10 amino acid deletion form of the TMV-MP were again remarkably similar to the values measured on the relevant control line. Note that these similarities held over the early afternoon, evening and

morning periods. Data obtained on plant lines 2004 and 3001 further confirmed that the elevated sugar and starch levels in TMV-MP transgenic plants can be attributed to the presence of the TMV-MP, rather than being due to unrelated events associated with plant transformation.

In this study, the influence of the TMV-MP on carbon metabolism and photosynthate export within source leaves of transgenic tobacco plants is further characterized. Earlier studies showed significantly higher levels of carbohydrates in mature source leaves of TMV-MP transgenic plants compared to control plants [Lucas et al. 1993, *supra*]. $^{14}CO_2$-labeling experiments were aimed at determining whether the reason for this high accumulation of sugars and starch was due to an alteration in export of newly fixed carbon from the source leaves of *TMV-MP* expressing plants. The results indicate that during the photoperiod, fully-expanded source leaves of plant lines 277 and 2004 exported ^{14}C at a lower rate than equivalently-aged control leaves (Fig. 1A, B). In addition, preliminary experiments performed on transgenic tobacco plants expressing both the TMV-MP and the TMV-coat protein yielded identical results, in that export was reduced during the day in the presence of the TMV-MP, but not in the presence of the TMV-coat protein.

Given that the sucrose levels are significantly different between TMV-MP expressing plants (lines 277, 2-72 and 2004) and control plants (lines 306 and 3001), it is possible that the differences in ^{14}C export could be accounted for in terms of differences in sucrose specific activity. The data presented in Table 8 were used to calculate the rate of ^{14}C-sucrose exported from the leaf. For these calculations it was assumed that, during the first hours after ^{14}C-labeling, all reductions in ^{14}C-sucrose were due to export via the phloem. It was also assumed that within the mature tobacco leaf sucrose is present in one pool in which newly synthesized ^{14}C-sucrose was able to mix with unlabeled sucrose. Based on these assumptions, the calculated values for ^{14}C-sucrose export from plant lines 277

and 306 were 13.6 and 15.3 $\mu g\ cm^{-2}\ h^{-1}$, respectively. It should be noted that the rate of reduction in the level of radioactivity in the liquid residue fraction (Table 8) was much greater in plant line 306 as compared with plant line 277, which suggests that the differences in ^{14}C-export between the two lines would actually have been larger than reflected by the above values. Furthermore, parallel measurements of photosynthesis and respiration established that these processes were occurring at equivalent rates in plant lines 277 and 306 (Table 9). Hence, the TMV-MP-induced alteration in ^{14}C turnover cannot be due to fundamental changes in either of these processes.

Experiments performed on young, expanding source leaves (leaf #2), in which the TMV-MP has yet to influence either plasmodesmal SEL [Deom et al. 1990, *supra*] or sugar levels [Lucas et al. 1993, *supra*] established that the presence of the TMV-MP also resulted in a small, but statistically significant, reduction in ^{14}C export in plant line 277 compared to control line 306. As the levels of sucrose would have been similar in these young leaves from lines 277 and 306 [Lucas et al. 1993, *supra*], the observed reduction in ^{14}C-export from 277 compared with 306 plants could not have been due to differences in the specific activity of ^{14}C-sucrose. These results are consistent with earlier studies performed on a different TMV-MP transgenic plant line (274), in which ^{14}C-labeling experiments established that, under the influence of the TMV-MP, young expanding source leaves partitioned lower amounts of ^{14}C-photosynthate to the lower stem and root tissues, compared with control plants [Lucas et al. 1993, *supra*]. Although the effect of the TMV-MP on export from these leaves is small, the long-term influence of this change in carbon allocation could well result in the observed phenotype of a reduced root-to-shoot ratio in *TMV-MP*-expressing, compared to control tobacco plants [Lucas et al. 1993, *supra*].

Sucrose is the major translocated sugar in many plant species, including tobacco [Giaquinta 1983, *supra*]. As

demonstrated by analysis of the data presented in Table 8, the actual turnover of ^{14}C in the sucrose pool is retarded by the presence of the TMV-MP. Similar trends were also detected for ^{14}C-glucose and ^{14}C-fructose, in that the absolute levels of these sugars were higher and the radiolabel was retained in TMV-MP transgenic, compared with control, plants (see also Table 10 and Fig. 2). Given that sucrose is confined mainly to the cytoplasm in wild-type tobacco mesophyll cells [Heineke, D., Wildenberger, K., Sonnewald, U., Willmitzer, L., Heldt, H.W. (1994) Accumulation of hexoses in leaf vacuoles: Studies with transgenic tobacco plants expressing yeast-derived invertase in the cytosol, vacuole or apoplasm. *Planta* **194**, 29-41], the TMV-MP may interact at any of the regulatory sites involved in controlling the compartmentation and/or the loading of sucrose into the sieve element-companion cell complex. Reduction in phloem loading would result in a rise in cytosolic sucrose which would lead to an increase in the level of fructose-6-phosphate. This change may then lead to an inhibition of triose phosphate export from the chloroplast which would result in increased partitioning of photosynthate into starch [Stitt, M., Quick, W.P. (1989) Photosynthetic carbon partitioning: its regulation and possibilities for manipulation. *Physiol. Plant.* **77**, 633-641]. Although this hypothesis can account for some of the observed effects of the TMV-MP on carbon metabolism and export, as will be pointed out below, the TMV-MP appears to act at additional target sites within the source leaf.

The influence of the TMV-MP on carbon metabolism and export in transgenic tobacco plants was further probed using a ts mutant form of the TMV-MP. The similarity in the results obtained on plant lines 2-72 and 277 (Fig. 2E, G and I), under permissive temperatures, provides further support for the hypothesis that the observed changes in carbon metabolism are caused by the presence of the TMV-MP. Under permissive temperatures (25°C), ^{14}C-export during the photoperiod was identical in plant lines 2-72 and 277 (Fig.

2A). Interestingly, in plants expressing the ts form of the *TMV-MP* gene, the level of starch was intermediate between the levels detected in lines 277 and 306 (Fig. 2C). However, under permissive temperatures, the ts and wild-type TMV-MPs elicited similar effects on sugar accumulation (Fig. 2E, G & I).

Under nonpermissive temperatures (32-34°C), the ts TMV-MP has no effect on plasmodesmal SEL [Wolf et al. 1991, *supra*]. Hence, if the influence of the TMV-MP on carbon metabolism were associated with an increase in plasmodesmal SEL, this effect should be negated in plants exposed to a 32-34°C regime. That this was not the case is established by the data presented in Fig. 2. For example, the level to which sucrose accumulated in the afternoon in plant line 2-72 was twice that measured in plant line 306 (Fig. 2F). The level in plant line 277 was intermediate between 2-72 and 306, which is of interest, as in these plants the plasmodesmata would have remained dilated by the TMV-MP (see also Table 8). Thus, the influence of the ts TMV-MP on sucrose levels strongly supports the hypothesis that the TMV-MP is pleiotropic in its effects within transgenic tobacco plants; i.e., this viral protein contains domains that allow it to interact with secondary plasmodesmata to potentiate the cell-to-cell transport of viral RNA [Lucas and Gilbertson 1994, *supra*; Waigmann et al. 1994, *supra*], as well as at least one domain that interferes with an endogenous process involved in regulating photoassimilate storage, translocation and partitioning [Huber, S.C, Huber, J.A.L., McMichael Jr., R.W. (1992) The regulation of sucrose synthesis in leaves. In *Carbon partitioning within and between organisms* (eds. Pollock, C.J., Farrar, J.F., Gordon, A.J.) pp. 1-26. BIOS Scientific Publishers, Ltd., Oxford]. Further evidence in support of the pleiotropic nature of the TMV-MP was provided by the finding that, under nonpermissive temperatures, the levels of glucose and fructose were always highest in plant line 2-72 compared with 277 and 306 (Fig. 2H & J).

Carbohydrate studies performed on transgenic tobacco

plants expressing a mutant form of the *TMV-MP* gene, in which the C-terminal 10 amino acids were deleted, provided strong additional support for the hypothesis that the TMV-MP has pleiotropic effects in source tobacco leaves. Although removal of this C-terminal region of the TMV-MP has no effect on its viral-related functions [Berna et al. 1991, *supra*; Gafny et al. 1992, *supra*], expression of this mutant form in tobacco plants (line MP1 and MP1-1) resulted in a return to normal photosynthetic carbon metabolism (Tables 9 & 10). Since the SEL of mesophyll secondary plasmodesmata, in these transgenic tobacco plants, would still be elevated to values greater than 9.4-kDa [Berna et al. 1991, *supra*], the results presented in Tables 9 and 10 clearly demonstrate that the TMV-MP has a second property (function), beyond that associated with dilating plasmodesmata, that allows it to interact with cellular processes involved in carbon metabolism and/or export (Fig. 3). In Fig. 3, T→ indicates control sites where the effect of the TMV-MP is overridden when sink strength is altered by raising the growth temperature from 24° to 32°C; PD, plasmodesmata; SEL, size exclusion limit.

Under the nonpermissive temperature regime used in the ts TMV-MP mutant studies, all plant lines tested behaved in an identical manner, in terms of net ^{14}C-export and starch metabolism (Fig. 2B & D). This result suggests that the interaction of the TMV-MP on the regulatory pathway(s) of photosynthate compartmentation and/or export is highly complex. Treating plants at high temperatures clearly induced a change in the source/sink balance. Under these elevated temperatures the vegetative apex entered into a phase of accelerated development, as evidenced by a striking increase in growth rate of all plant lines tested (data not shown). As expected, high temperatures also caused a significant increase in dark respiration (Table 9). These temperature-induced changes in the apical tissues reflect a substantial increase in overall sink strength, which resulted in enhanced sucrose transport from the source leaves, thereby

reducing carbohydrate levels in the mesophyll (Fig. 2). Thus, the temperature-induced convergence of net carbon export from lines 2-72, 277 and 306 (see Fig. 2B) may reflect the influence of an hierarchical control site, involving a feedback (or feedforward) mechanism whose input signal can override some of the sites where the TMV-MP interacts with the endogenous regulatory pathway(s) that controls photosynthate export and partitioning (see Fig. 3).

In this regard it is interesting to note that, under the high temperature regime, the levels of starch were almost identical within the mature source leaves of plant lines 2-72, 277 and 306. Thus, increased sink demand for photosynthate can completely override the effects of the TMV-MP on altered starch metabolism. However, as the levels of sucrose, glucose and fructose remained elevated in the ts TMV-MP plants (line 2-72), it may well be that there are numerous sites at which the TMV-MP can influence (alter) carbon metabolism.

Example 3

It is well established that control over developmental process in plants and animals is orchestrated, at the cellular level, by transcription factors, one class of which is called homeodomain proteins [Gehring WJ. (1987) Homeo boxes in the study of development. *Science* **236**, 1245-1252; Desplan C., Theis J., O'Farrell P.H. (1988) The sequence specificity of homeodomain-DNA interactions. *Cell* **54**, 1081-1090]. In *Zea mays, Knotted* encodes a homeodomain protein which is expressed at very low levels in leaves [Vollbrecht E., Kerstetter R., Lowe B., Veit B., Hake S. (1991) The developmental gene *Knotted-1* is a member of a maize homeobox gene family. *Nature* **350**, 241-243; Smith L., Greene B., Veit B., Hake S. (1992) A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates. *Development* **116**, 21-30], but at high levels in the meristem and ground tissue of unexpanded stems [Vollbrecht et al. 1991, *supra*; Jackson D., Veit B., Hake S.

(1994) Expression of maize *KNOTTED1* related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. *Development* **120**, 405-413]. It has also been established that ectopic expression of *Knotted* in young leaves allows cells that would normally be determinate in nature to undergo continued cell division, resulting in aberrant morphology [Hake S., Freeling M. (1986) Analysis of genetic mosaics shows that the extra epidermal cell divisions in *Knotted* mutant maize plants are induced by adjacent mesophyll cells. *Nature* **320**, 621-623; Smith et al. 1992, *supra*).

In the normal situation, as well as in tissues where *Knotted* is expressed ectopically, it appears that the mRNA for *Knotted* is synthesized in one tissue while the protein, KNOTTED, is detected within the nuclei of cells located within the surrounding tissue(s) [Jackson et al. 1994, *supra*]. This observation is consistent with the hypothesis that plasmodesmal transport of transcription factors plays a central role in the orchestration of plant development. In Table 12 experimental evidence is presented that establishes the validity of this concept. Here, wild-type protein of KNOTTED (KN1) was fluorescently labelled and then microinjected into target cells within intact plants. As with earlier studies on viral movement proteins [Lucas and Gilbertson 1994, *supra*], evidence was obtained consistent with KN1 having the capacity to interact with plasmodesmata to mediate in its own cell-to-cell transport. Although some mutant forms of KNOTTED could still move out of the target cell into the surrounding tissues, one mutant, KN1 M11Y51, was fully defective in transport function (Table 12). This mutant serves the essential function of an internal control for these experiments.

Having established that KN1 has the ability to mediate in its own cell-to-cell transport, whether KN1 has the ability to interact with its own mRNA to allow the mRNA to undergo transport from the site of synthesis to the cells where KN1 controls tissue development was next tested. As

illustrated in Table 13, coinjection of KN1 and fluorescently labelled *Knotted* mRNA resulted in the efficient transport of mRNA from the target cell into the cells of the surrounding tissues. As expected, injection of fluorescently labelled *Knotted* mRNA alone resulted in the confinement of the fluorescent probe to the injected cell (Table 13). Further, coinjection of the nonfunctional KN1 M11Y51 mutant and fluorescently labelled *Knotted* mRNA also resulted in the mRNA being confined to the target cell (Table 13). Collectively, these results provide incontrovertible proof that KN1, a plant-encoded protein, engages in its own cell-to-cell transport as well as the transport of its mRNA. As KNOTTED is a member of a large gene family [Vollbrecht E., Kerstetter R., Lowe B., Veit B., Hake S. (1993) Homeobox genes in plant development: Mutational and molecular analysis. In: *Evolutionary Conservation of Developmental Mechanisms* (ed. A.C. Spradling) pp. 111-123, New York, Wiley-Liss; Jackson et al. 1994, *supra*], it is highly likely that this ability for macromolecular transport, via plasmodesmata, is central to the control over developmental processes. Identical results have also been obtained with the transcription factors encoded by the MADS box genes *deficiens* and *globosa* of Antirrhinum. Microinjection studies clearly established that both of these transcription factors have the capacity to interact with the supramolecular complex of the plasmodesmata to mediate in their cell-to-cell transport.

Example 4

Morphological features as well as biomass partitioning vary as a function of the environmental conditions under which control, wild-type and deletion mutant forms of the TMV-MP transgenic plants are grown [Atkins et al. 1993 *supra*; Balachandran S., Hull R.J., Vaadia, Y., Wolf S., Lucas, W.J. (1995) Alteration in carbon partitioning, induced by the movement protein of tobacco mosaic virus, originates from the mesophyll and is independent of change in plasmodesmal size exclusion limit. *Plant, Cell & Environment*, 1301-10]. In

general, these TMV-MP expressing transgenic tobacco plants exhibit varying degrees of reduction in both plant height and root biomass. Growth characteristics for control, wild-type TMV-MP and an N-terminal deletion mutant (Mn-5) are presented in Table 14. Plant line Mn-5 (expressing a mutant TMV-MP in which 3 amino acids [#3-#5] from the N terminal were deleted [Lapidot et al. 1993 *supra*.]) exhibited the most striking phenotype when grown under high light conditions. Plant height as well as total dry weight were approx. 40 and 50 percent lower than those of plant lines 277 (wild type) and 306 (control), respectively. Interestingly, mean internodal length was not significantly different between the three plant lines. It is important to note that the root-to-shoot ratio of plant line Mn-5 was similar to that of plant line 277, despite its specific phenotype. Finally, plants expressing either wild-type or an N-terminal deletion TMV-MP always had lower root-to-shoot ratios compared to control plants (Tables 2 and 14).

Under limiting light conditions, TMV-MP transgenic and vector control plants undergo a significant reduction in root-to-shoot ratio (Table 14). The important point to note is that under these light conditions all plant lines tested had equivalent root-to-shoot ratios; i.e., in the presence of limiting light, the endogenous control system(s) involved in biomass partitioning appears to override the influence of the TMV-MP. However, the presence of the TMV-MP still appeared to influence other developmental processes. For example, while control plants responded to low light conditions by increasing the mean internodal length, which gave rise to an elongation of the stem, a differential growth response was observed in plants expressing the TMV-MP. Mean internodal length increased significantly in plant line 277, but without a concomitant increase in plant height, due to a decrease in the number of nodes produced. Deletion of 3 amino acids within the N-terminus of the TMV-MP (plant line Mn-5) resulted in a complete lack of a response to the low light conditions. Both the number of internodes and the mean

internodal length were similar under either 1200 or 150 μmol m^{-2} s^{-1} of irradiance (Table 14). This differential response to low light conditions, observed with wild-type and N-terminal deletion mutant TMV-MP, suggests that the TMV-MP may interact (interfere) with elements of a phytochrome signal transduction cascade.

Example 5

Cell fate in higher plants is determined by position, rather than by lineage [E. Huala, I. M. Sussex, *Plant Cell* **5**, 1157 (1993); I. M. Sussex, *Cell* **56**, 225 (1989)]. Although environmental and hormonal signals could act in a cell autonomous manner to control cell fate, clonal analysis of developmental mutants has indicated that cell-to-cell transport may be involved in the orchestration of developmental events [W. J. Lucas, *Curr. Opinion Cell Biol.* **7** 673-680]. For example, expression of *Floricaula* (FLO), which affects meristem identity in *Antirrhinum majus* (snapdragon), in only the outer (epidermal) layer (L1) of the meristem, activates down-stream genes involved in flower development [R. Carpenter, E. S. Coen, *Development* **121**, 19 (1995); S. S. Hantke, R. Carpenter, E. S. Coen, *ibid* **121**, 27 (1995)] in adjacent cell layers. Similarly, the genotype of the inner layer (L3) of the tomato floral meristem controls development of the outer layers (L2 and L1) [E. J. Symkowiak, I. M. Sussex, *Plant Cell* **4**, 1089 (1992)]. These findings are consistent with the hypothesis that FLO, and the fasciated gene product of tomato, potentiate cellular interactions between the three layers of the floral meristem. Such control may involve the selective cell-to-cell transport of proteins through plasmodesmata.

An analysis of movement of the protein and RNA encoded by the maize *knotted1* (*kn1*) homeobox gene [S. Hake, M. Freeling, *Nature* **320**, 621 (1986); N. Sinha, S. Hake, *Dev. Biol.* **141**, 203 (1990); E. Vollbrecht, B. Veit, N Sinha, S. Hake, *Nature* **350**, 241 (1991); L.G. Smith, B. Greene, B. Veit, S. Hake, *Development* **116**, 21 (1992)] is here reported.

Ectopic expression of *knl* in the vascular tissue of developing maize leaves alters cell differentiation within adjacent mesophyll and epidermal layers, suggesting that a signal moves from one cell layer to another. *In situ* and immunolocalization studies of the maize shoot apical meristem demonstrated that *knl* mRNA was detected only within the interior (L2) cells of the meristem, whereas KN1 was detected in the L2 cells and in the epidermal (L1) layer [D. Jackson, B. Veit, S. Hake, *Development* **120**, 405 (1994)].

Serial sections of a maize vegetative shoot apex, processed for *in situ* hybridization for *knl* mRNA and immunolocalization of KN1, revealed the presence of KN1 in L1 cells in which its mRNA was not detected. The shoot apical meristem was flanked by leaf primordia and older expanding leaves in which *knl* mRNA and KN1 were not detected. Regions in the shoot apical meristem that lacked KN1 predicted the position of leaf primordial development. KN1 was present in a few cells across the base of each developing leaf.

These results suggested that, despite the fact that KN1 is a nuclear-localized transcription factor, it is likely the signal that is transported from L2 into the L1 layer, as well as between cell layers in knotted leaves.

In situ hybridization and immunolocalization experiments were performed on paraffin-embedded maize seedling apices. *In situ* hybridization was performed exactly as described by D. Jackson, B. Veit and S. Hake in *Development* **120**, 405 (1994), while for immunolocalization the method of L.G. Smith, B. Greene, B. Veit and S. Hake in *Development* **116**, 21 (1992) was used, except that tissue was embedded in paraffin wax and sections were predigested with proteinase K (Sigma), at 100 µg/ml in PBS, for 10 min at room temperature and then rinsed twice in PBS before the blocking step. Goat anti-rabbit-alkaline phosphatase (Boehringer Mannheim) was used as the secondary antibody (1:600 dilution) and visualized according to Jackson et al. [D. Jackson, B. Veit, S. Hake, *Development* **120**, 405 (1994)]. Sections were lightly counterstained in basic fuchsin (0.005 % w/v).

In another study, fluorescently-labeled *Escherichia coli*-expressed KN1 (FITC-KN1) was microinjected into the cytoplasm of plant cells. Wild-type and mutant KN1 were expressed, extracted and labeled with fluorescein isothiocyanate (FITC) using our procedures developed for viral movement proteins [T. Fujiwara, D. Giesman-Cookmeyer, B. Ding, S. A. Lommel, W. J. Lucas, *Plant Cell* **5**, 1783 (1993); A. O. Noueiry, W. J. Lucas, R. L. Gilbertson, *Cell* **76**, 925 (1994); E. Waigmann, W. J. Lucas, V. Citovsky, P. Zambryski, *Proc. Natl. Acad. Sci. (USA)* **91**, 1433 (1994); B. Ding, L. Qiubo, L. Nguyen, P. Palukaitis, W. J. Lucas, *Virology* **207**, 345 (1995)]. As an internal control, proteins were extracted and FITC-labeled from an *E. coli* preparation which did not contain the *kn1* cDNA. Alanine scanning mutants were created in groups of charged amino acids, which are likely to be present in surface domains (PC gene software, Intelligenetics). The *kn1* cDNA (BamHI-NcoI partial digest) from pKOC10 was inserted into the pET23-d(+) vector (Novagen) to create pDJX-1. Single-stranded virions were produced in the CJ236 (*dut ung*) strain of *E. coli*, and site-directed mutagenesis was performed using oligonucleotides of 33-48 bases and T7 DNA polymerase, according to the manufacturer's instructions (United States Biochemical). Mutagenized clones were confirmed by sequencing before transfer to strain BL21(DE3) for protein production. Microinjections were carried out essentially as previously described [S. Wolf, C. M. Deom, R. N. Beachy, W. J. Lucas, *Science* **246**, 377 (1989)], except for the modifications noted in the above citations to procedures developed for viral movement proteins.

The small size of cells in the maize shoot apical meristem precluded us from performing microinjection experiments on such tissues. Instead, using developing maize leaves, microinjections were made into mesophyll cells connected to the vascular bundle, as this was the site where ectopically expressed *kn1* was shown to alter cell fate. [S. Hake, M. Freeling, *Nature* **320**, 621 (1986); N. Sinha, S. Hake, *Dev. Biol.* **141**, 203 (1990); E. Vollbrecht, B. Veit, N Sinha,

S. Hake, *Nature* **350**, 241 (1991); L.G. Smith, B. Greene, B. Veit, S. Hake, *Development* **116**, 21 (1992)]. FITC-KN1 injected into the cytoplasm of these mesophyll cells moved into bundle sheath and surrounding mesophyll cells (Table 15). Thus, KN1 must be capable of interacting with plasmodesmata to potentiate its own movement from cell to cell.

Tobacco offers another system in which to study KN1, as ectopic meristems are also obtained when KN1 is overexpressed in tobacco [N. Sinha, R. Williams, S. Hake, *Genes & Dev.* **7**, 787 (1993)]. FITC-KN1 microinjected into mesophyll cells of tobacco (*Nicotiana tabacum* cv. Samsun) leaves also moved to neighboring cells. See Table 16. Just as an increase in plasmodesmal size exclusion limit (SEL) is required for cell-to-cell transport of viral movement proteins [W. J. Lucas, R. L. Gilbertson, *Annu. Rev. Phytopath.* **32**, 387 (1994); T. Fujiwara, D. Giesman-Cookmeyer, B. Ding, S. A. Lommel, W. J. Lucas, *Plant Cell* **5**, 1783 (1993); A. O. Noueiry, W. J. Lucas, R. L. Gilbertson, *Cell* **76**, 925 (1994); E. Waigmann, W. J. Lucas, V. Citovsky, P. Zambryski, *Proc. Natl. Acad. Sci. (USA)* **91**, 1433 (1994); B. Ding, L. Qiubo, L. Nguyen, P. Palukaitis, W. J. Lucas, *Virology* **207**, 345 (1995)], an increase in plasmodesmal SEL is also associated with KN1 cell-to-cell movement in maize and tobacco (Tables 15 and 16).

In an experiment focusing on cell-to-cell transport of FITC-labeled KN1 and its effect on plasmodesmal SEL in tobacco mesophyll cells, KN1 and its mutant derivative, M6 (see Fig. 4), were expressed in *E. coli* and extracted proteins were labeled with FITC prior to being used in microinjection studies. Immediately after being introduced into a tobacco mesophyll cell FITC-labeled KN1 moved into surrounding cells as indicated by false-color imaging obtained with a Hamamatsu model C1966 analytical system. Containment of FITC-labeled M6 was observed 15 minutes after injection into the cell. Injected 20 kDa FITC-dextran remained indefinitely (60 minutes after injection) within the

target cell. Coinjection of 20 kDa FITC-dextran and unlabeled KN1 resulted in extensive movement after 2 minutes after injection.

Cell-to-cell movement of injected Lucifer yellow CH (MW 457), a membrane-impermeant fluorescent probe, established that plasmodesmata in the injected tissues displayed normal characteristics [A. W. Robards, W. J. Lucas, *Annu. Rev. Plant Physiol. Plant Molec. Biol.* **41**, 369 (1990)] (Tables 15 and 16). Furthermore, the lack of movement of FITC-labeled bacterial proteins established that KN1 movement was not an artifact of the preparative techniques employed (Table 16).

Microinjection of 9.4 or 20 kDa FITC-dextran alone did not result in movement out of the injected cell, but coinjection of these FITC-dextrans with unlabeled KN1 gave rise to the same spread of fluorescence as detected when FITC-KN1 was introduced into the cell (Tables 15 and 16). This KN1-induced increase in plasmodesmal SEL also permitted the cell-to-cell movement of a labeled, coinjected, 20 kDa soybean cytosolic protein, soybean trypsin inhibitor (Table 16). Occasionally, KN1 permitted the movement of a 39 kDa FITC-dextran, and so the upper plasmodesmal SEL associated with KN1 transport is greater than 20 and close to 39 kDa.

Protein domains essential for KN1 cell-to-cell movement were investigated using a series of alanine scanning mutants (created as described above). (Fig. 4). Of the 9 mutants studied, only one (M6) showed a significant reduction in ability to move from cell to cell (Table 16). The M6 mutation resides in a potential nuclear localization sequence present in the N-terminal region of the homeodomain [E. Vollbrecht, R. Kerstetter, B. Lowe, B. Veit, S. Hake, in *Evolutionary Conservation of Developmental Mechanisms*, A. C. Spalding, Ed. (Wiley-Liss, New York, 1993), 111; R. Kerstetter, E. Vollbrecht, B. Lowe, B. Veit, J. Yamaguchi, S. Hake, *Plant Cell* **6**, 1877 (1994); C. Lincoln, J. Long, J. Yamaguchi, K. Serikawa, S. Hake, *Plant Cell* **6**, 1859 (1994)]. Whether this reflects homology between nuclear and plasmodesmal transport systems must await the identification and characterization of

other transcriptional regulators that also have the capacity for plasmodesmal transport.

Although the other mutants of KN1 retained the capacity to dilate plasmodesmata and potentiate their own cell-to-cell transport (Table 16), the rate and extent of movement of each mutant KN1 was reduced, compared to wild-type KN1. FITC-KN1 was routinely detected in neighboring cells one to two seconds after its injection into a mesophyll cell, with further movement through five to ten surrounding cells in approx. 30 seconds. Although the period before each mutant FITC-KN1 could be detected in the neighboring cells was also short (a few seconds), subsequent movement into the second layer of cells required from 3 to 5 minutes. Furthermore, rarely was fluorescence detected beyond this second layer of mesophyll cells. Analysis of plant viral movement proteins (by procedures described and cited above), on the other hand, showed that alanine scanning mutants either exhibited normal movement, or were incapable (0% movement) of cell-to-cell transport. The varied response of KN1 mutants may reflect the presence of multiple domains involved in mediating efficient plasmodesmal transport or interaction with the plasmodesmata.

Having established that KN1 interacts with plasmodesmata to increase SEL and mediate in its own cell-to-cell transport, characteristics held in common with many viral movement proteins, it was next investigated whether KN1 could also mediate trafficking of nucleic acids, although the results above suggest no such ability. Sense *kn1* RNA was TOTO-labeled and coinjected into mesophyll cells with unlabeled KN1. *Kn1* sense or antisense RNA was transcribed using T3 or T7 RNA polymerase from linearized pKOC10 plasmid which contained the full length cDNA. The DNA template was digested with RQ1 DNase (Promega) and the RNA was phenol extracted and ethanol precipitated. *Kn1* RNA (1.6 kb) was resuspended in 20 μl DEPC-H_2O and concentration and purity was determined by spectroscopy. Sense and antisense RNA (500 μg/ml) were labeled with the nucleotide-specific fluorescent probe, TOTO-1 (Molecular Probes), as previously described.

All *kn1* RNA-TOTO preparations were adjusted to 225μg/ml for use in microinjection experiments. CMV RNA-TOTO was adjusted to 250 - 500 μg/ml.

In the presence of KN1, the fluorescence associated with *kn1* sense RNA-TOTO moved as rapidly and extensively from cell-to-cell as did FITC-KN1 when it alone was injected into this tissue. Control microinjection experiments, involving *kn1* sense RNA-TOTO alone, *kn1* antisense RNA-TOTO alone, or unlabeled KN1 plus *kn1* antisense RNA-TOTO, established the specificity of KN1-mediated *kn1* RNA transport, as in each of these cases, the fluorescent probes remained in the injected cell (Table 17). The M6 mutant of KN1, which was least able to transport itself, did not potentiate the cell-to-cell transport of *kn1* sense RNA-TOTO (Table 17).

Coinjection of KN1 and *kn1* sense RNA-TOTO revealed movement of the *kn1* sense RNA-TOTO into tobacco mesophyll cells within the vicinity of the injected cell after 1 minute. *Kn1* antisense RNA-TOTO failed to move out of the target cell when coinjected with KN1. At 15 minutes after coinjection, a false-color image showed that fluorescence had accumulated in what appeared to be the nucleus. A tobacco mesophyll cell coinjected with KN1 and CMV RNA-TOTO after 15 minutes showed fluorescence remains confined to the injected cell. Purified CMV RNA was prepared [P. Palukaitis, M. Zaitlin, *Virology* **132**, 426 (1984)] and TOTO-labeled as described by Ding et al. This preparation contained 3 single-stranded RNA species, RNA1 (3.3 kb), RNA2 (3.0 kb) and RNA3 (2.2 kb). The procedures of Ding et al. were used to prepare and FITC-label the CMV 3a movement protein. Although KN1 would presumably have trafficked into surrounding cells, it failed to transport the CMV RNA-TOTO. Coinjection of CMV 3a movement protein and *kn1* sense RNA-TOTO into a tobacco mesophyll cell resulted in extended CMV 3a movement protein-mediated transport of the *kn1* sense RNA-TOTO into the surrounding cells, 2 minutes after injection.

KN1 was selective in terms of the RNA that it would traffic as shown by coinjection of TOTO-labeled cucumber

mosaic virus (CMV) single-stranded sense RNA and KN1 (Table 17). The CMV movement protein, in contrast, potentiated cell-to-cell transport both of its own RNA and of *kn1* RNA (Table 17), consistent with the known non-specificity of viral movement proteins.

Our finding that KN1 has the capacity to move from cell to cell provides a plausible explanation for the non-cell autonomy of the dominant *Kn1* mutation, as well as the lack of autonomy found with other developmental mutations [P. W. Becraft, M. Freeling, *Genetics* **136**, 295 (1994)].

These studies on KN1 provide important insights into the molecular events that orchestrate developmental processes in plants and establish a conceptual basis for explaining the plasticity of cell fate in the plant meristem.

From the foregoing description, one skilled in the art can readily ascertain the essential characteristics of the invention and, without departing from the spirit and scope thereof, can adapt the invention to various usages and conditions. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient, and although specific terms have been employed herein, they are intended in a descriptive sense and not for purposes of limitation.

Each and every reference cited herein is, in its entirety, expressly incorporated by reference into the foregoing disclosure.

Table 1. Description of *TMV-MP* and various mutant forms of the *TMV-MP* used to examine the influence of the MP on carbon allocation in transgenic tobacco plants expressing these genes.

Transgenic line	Type of MP gene expressed	Reference[a]
277	*TMV-MP* (wild-type) (*N. tabacum* Xanthi nn)	Deom et al. (1987)
306	Vector control, (plasmid without *MP* ; Xanthi nn)	Deom et al. (1987)
MN-1	33 amino acid deletion in the C-terminus of *TMV-MP* , (Xanthi NN)	Berna et al. (1991); Gafny et al. (1992)
MN-2	55 amino acid deletion in the C-terminus of *TMV-MP* , (Xanthi NN)	Berna et al. (1991); Gafny et al. (1992)
Mn-1	10 amino acid deletion in the C-terminus of *TMV-MP* , (Xanthi nn)	Berna et al. (1991); Gafny et al. (1992)
Mn-2	10 amino acid deletion in the C-terminus of *TMV-MP* , (Xanthi nn)	Berna et al. (1991); Gafny et al. (1992)
Mn-3	73 amino acid deletion in the C-terminus of *TMV-MP* , (Xanthi nn)	Berna et al. (1991); Gafny et al. (1992)
Mn-4	116 amino acid deletion in the C-terminus of *TMV-MP* , (Xanthi nn)	Lapidot et al. (1993)
Mn-5	3-5 amino acid deletion in the N-terminus of *TMV-MP* , (Xanthi nn)	Lapidot et al. (1993)
Mn-6	Same as Mn-5, (no protein detected even though *MP* was present)	Berna et al. (1991); Gafny et al. (1992)
3001	Vector control, (plasmid without *MP* , Xanthi NN)	Deom et al. (1991)
2005	*TMV-MP*, (Xanthi NN)	Deom et al. (1991)
RMN-1	*TMV-MP* expressed in phloem and hair tip cells, (under *rolC* promoter in Xanthi NN)	Reimann-Philipp & Beachy (1993)
RMn-1	*TMV-MP* expressed in phloem and hair tip cells, (under *rolC* promoter in Xanthi nn)	Reimann-Philipp & Beachy (1993)
RGN-1	Vector control, (plasmid without *MP* expressed in phloem and hair tip cells (under *rolC* promoter in Xanthi NN))	Reimann-Philipp & Beachy (1993)

a See primary reference for details relating to plasmid construction and plant transformation.

Table 2. Root-to-shoot ratios obtained from experiments performed on grafted transgenic tobacco plants expressing either the TMV-MP (line 277) or the vector control (plasmid only; line 306).

Scion/stock	R/S[a]
277/306	0.07 ± 0.008[b,c]
306/277	0.12 ± 0.001[b,d]
277/277	0.06 ± 0.005[c]
306/306	0.10 ± 0.007[d]

[a] Root-to-shoot ratios expressed as a fraction of the root biomass divided by total above-ground plant material. Values represent mean ± SE (n= 3-8).
b, significantly different at P<0.01; c, d not significantly different at P=0.05

Table 3. Biomass partitioning in transgenic tobacco lines in which the TMV-MP was express expressed in different plant tissues. (Data represent one of two sets of identical, indepently run experiments. Values are mean ± SE,n=5-10.)

Transgenic line	Dry weight (g)[a]				
	Total plant weight	Leaves	Stems	Roots	R/S
306	26.4±2.9[a,b]	12.5 ± 1.4[e]	11.8 ± 1.3[f,g]	2.1 ± 0.3 [a,j]	0.09 ± 0.004 [j,k]
277	20.7±1.5[a,c,d]	12.2 ± 1.1[e]	7.4 ± 0.5[f]	1.0 ± 0.1 [h]	0.05 ± 0.001 [j]
RMn-1	28.4±2.6[b,c]	13.1 ± 0.8[e]	12.7 ± 1.3	2.6 ± 0.2 [i]	0.10 ± 0.005 [k]
RGN-1	23.7±1.5[b,d]	12.3 ± 0.5[e]	9.5 ± 0.6	1.9 ± 0.1 [i]	0.09 ± 0.002 [k]
RMN	21.9±2.0 [b,d]	11.0 ± 1.1[e]	9.9 ± 0.9 [g]	2.0 ± 0.2 [i]	0.10 ± 0.002 [k]

[a], Plants were 50 days old at time of harvest. Values represent mean ± SE, n= 6.

b, significantly different at P<0.01, c, not significantly different at P=0.05.

Table 4. Biomass partitioning in transgenic tobacco lines expressing different deletion mutant forms of the TMV-MP. Data represent one of two sets of identical, independently run experiments.

Transgenic line		Dry weight (g)[a]				
		Total plant weight	Leaves	Stems	Roots	R/S
Xanthi NN	3001	2.5 ± 2.2	10.5 ± 0.9	10.2 ± 1.1[g]	1.9 ± 0.2	0.09 ± 0.004[b,d]
	2005	17.6 ± 1.6	11.8 ± 0.9	5.3 ± 0.7	0.6 ± 0.1	0.03 ± 0.004[b,f]
	MN-1	14.8 ± 1.2	9.2 ± 0.9	5.1 ± 0.5	0.6 ± 0.1	0.04 ± 0.004[f]
	MN-2	19.1 ± 2.1	10.0 ± 0.7	8.1 ± 1.3[g]	1.0 ± 0.2	0.05 ± 0.008[f]
Xanthi nn	306	26.4 ± 2.9	12.5 ± 1.4	11.8 ± 1.3	2.1 ± 0.3	0.09 ± 0.004[c,d]
	277	20.7 ± 1.5	12.2 ± 1.1[j]	7.4 ± 0.5[h]	1.0 ± 0.1[i]	0.05 ± 0.001[c,e]
	Mn-3	18.5 ± 1.8	9.1 ± 0.5	8.4 ± 0.6	1.0 ± 0.1	0.06 ± 0.008[e]
	Mn-4	21.9 ± 0.8	10.4 ± 2.2	10.4 ± 0.4	1.3 ± 0.4	0.06 ± 0.006[e]
	Mn-5	10.3 ± 2.7	5.8 ± 1.3[j]	3.8 ± 1.0[h]	0.6 ± 0.1[i]	0.07 ± 0.003[e]
	Mn-6	25.2 ± 0.4	11.3 ± 0.2	11.9 ± 0.5	2.0 ± 0.2	0.09 ± 0.003[d]

[a], Plants were 70-75 days old at time of harvest. Values represent mean ± SE, n= 5-10.

b,c,g, significantly different at P< 0.01; h, i, j, significantly different at P< 0.05; d, e, f, not significantly different at P=0.05.

Table 5. Growth characteristics of transgenic tobacco plants expressing different deletion mutant forms of the TMV-MP. Data represent one of two sets of identical, independently run experiments.

Transgenic line		Growth characteristics[a]	
		Plant height (cm)	Number of leaves
Xanthi NN	3001	41 ± 1.5 [b,i]	18 ± 0.5 [i]
	2005	27 ± 1.5 [b,c]	18 ± 0.5 [i]
	MN-1	27 ± 1.7 [c]	17 ± 0.6 [i]
	MN-2	37 ± 1.1 [f]	20 ± 0.4 [i]
Xanthi nn	306	46 ± 0.9 [d,e]	19 ± 1.1 [g,h,j]
	277	29 ± 1.1 [c,d]	17 ± 0.7 [i]
	Mn-3	33 ± 1.5 [c]	16 ± 1.8 [g]
	Mn-4	33 ± 0.5 [c]	16 ± 0.6 [i]
	Mn-5	26 ± 2.8 [c]	15 ± 1.1 [h]
	Mn-6	47 ± 1.6 [e]	19 ± 0.4 [i]

[a], Plants were 70-75 days old at time of harvest. Values represent mean ± SE, n= 5-10. b, d, f, g, h significantly different at P< 0.05; c, e, i not significantly different at P= 0.05.

Table 6. Biomass partitioning in transgenic tobacco lines infected with tobacco mosaic virus (strain PV230). Plants were inoculated with TMV when they had six fully developed leaves, and were harvested at 24 days post-inoculation at which time they were 60 days old. (Values represent mean ± SE, n= 4.)

Transgenic line	Dry weight (g)				
	Total plant weight	Leaves	Stems	Roots	R/S
306 unifected	45.6 ± 3.1	21.5 ± 1.0	19.5 ± 2.1	4.7 ± 0.3	0.11 ± 0.004[a]
infected	40.3 ± 1.0	18.6 ± 0.4	19.1 ± 1.2	2.5 ± 0.1	0.07 ± 0.003[a,b]
277 unifected	31.8 ± 1.0	17.0 ± 0.8	12.7 ± 0.5	2.1 ± 0.2	0.07 ± 0.005[b]
infected	34.7 ± 1.5	18.3 ± 0.7	14.0 ± 0.9	2.3 ± 0.2	0.07 ± 0.003[b]

a, significantly different at P<0.01, b, not significantly different at P=0.05

Table 7. Biomass partitioning in transgenic tobacco lines (Mn-1 and Mn-2) expressing the TMV-MP in which 10 amino acids were deleted from the C-terminus. (Note that Mn-1 and Mn-2 represent two independent transformed lines.)

Transgenic line	Dry weight (g)[a]				
	Total plant weight	Leaves	Stems	Roots	R/S
306	26.2 ± 2.4	12.2 ± 0.7	11.4 ± 0.8	2.6 ± 0.1	0.11 ± 0.007 [b,c]
277	19.3 ± 2.0	11.4 ± 0.7	6.7 ± 0.9	1.2 ± 0.1	0.07 ± 0.008 [b]
Mn-1	26.4 ± 2.0	12.3 ± 0.7	11.7 ± 0.9	2.4 ± 0.2	0.10 ± 0.006 [c]
Mn-2	27.1 ± 2.8	12.5 ± 0.7	11.8 ± 0.6	2.8 ± 0.3	0.12 ± 0.01 [c]

[a], Plants were 50 days old at time of harvest. Values represent mean ± SE, n= 6.

b, significantly different at P<0.01, c, not significantly different at P=0.05.

Table 8. ^{14}C-carbohydrates and total carbohydrate content within leaves of TMV-MP transgenic (line 277) and vector control (line 306) tobacco plants. Plants were grown in a greenhouse under natural sunlight with average midday photon flux density of 1500 $\mu mol.m^{-2}.s^{-1}$. Six plants were used from each line. Leaf disks were punched from fully expanded leaves (leaf #5-6), and were analyzed for radioactivity and carbohydrate content (values in parentheses) of each compound.

Plant line	Time of day	Radioactivity[a] and content										
		SC[b]		GL		FR		STR		LR	SR	TOT
306	10:30	368	(28.7)	161	(38.0)	138	(54.2)	506	(462)	533	123	1829
	14:00	120	(37.7)	158	(43.7)	183	(54.3)	546	(577)	319	133	1459
	17:30	81	(31.5)	117	(36.4)	136	(52.5)	505	(659)	255	152	1244
Significance[c]		*	*	ns	ns	ns	ns	ns	*	*	*	*
277	10:30	479	(47.6)	176	(92.4)	148	(114.5)	631	(1002)	564	128	2127
	14:00	293	(61.4)	171	(101.6)	173	(125.8)	579	(1041)	535	151	1902
	17:30	227	(59.0)	168	(95.4)	185	(118.5)	573	(1076)	519	169	1841
Significance[b]		*	*	ns	ns	ns	ns	ns	ns	ns	*	*

[a] Radioactivity and content (values in parenthesis) presented as DPM and $\mu g.cm^{-2}$, respectively.

[b] SC, sucrose; GL, glucose; FR, fructose; STR, starch; LR, liquid residue; SR, solid residue; TOT, total radioactivity

[c] * and ns represent significant and not significantly different, respectively, at P=0.05.

Table 9. Temperature effects on photosynthesis and dark respiration in wild-type TMV-MP transgenic (line 277), ts mutant TMV-MP transgenic (line 2-72) and control (line 306) tobacco plants. Mature leaves (leaf #5-6 on 12 to 14-leaf plants) were measured using a closed infrared gas-exchange system. (Six plants of each line were used per experiment and value presented represent mean ± SE.)

Plant line	Temperature (°C)	Plasmodesmal[a] SEL (kDa)	Photosynthesis	Dark respiration
			μmol $CO_2.m^{-2}.s^{-1}$	
306	25	0.8	14.4 ± 0.9	2.6 ± 0.2
277	25	>9.4	12.5 ± 1.0	2.4 ± 0.2
2-72	25	>9.4	14.1 ± 3.3	2.2 ± 0.2
306	32-34	0.8	23.8 ± 1.3	6.1 ± 0.4
277	32-34	>9.4	24.6 ± 0.2	5.9 ± 0.4
2-72	32-34	0.8	22.3 ± 0.9	6.2 ± 0.7

[a] Data from Wolf et al. (1991)

Table 10. Carbohydrate content within source leaves of transgenic tobacco plants expressing wild-type TMV-MP (line 277), a 10 amino acid C-terminal deletion mutant of the TMV-MP (line MP1), and the vector control (line 306). Experimental details as in Table 8 (mean ± SE, n = 5).

Time of day	Plant line	Sucrose[a]	Glucose	Fructose	Starch[b]
13:00	277	125.6 ± 12.7 d	236.7 ± 46.4 d	172.5 ± 33.6	817 ± 51 d
	MP1	61.7 ± 16.8 c	143.3 ± 26.8 c	126.5 ± 23.7	521 ± 40 c
	306	65.7 ± 11.5 c	129.4 ± 12.9 c	130.4 ± 12.9	528 ± 54 c
Significance[c]		**	*	ns	**
19:00	277	177.1 ± 8.7 d	194.3 ± 45.9 d	162.6 ± 36.2 d	979 ± 146
	MP1	61.9 ± 14.4 f	105.1 ± 20.5 c	102.5 ± 15.6 c	854 ± 103
	306	115.9 ± 7.3 c	106.9 ± 14.6 c	126.3 ± 14.5 dc	758 ± 74
Significance		**	*	*	ns
07:00	277	52.5 ± 9.3 d	169.9 ± 37.4 d	129.4 ± 21.4 d	954 ± 151d
	MP1	5.5 ± 4.3 c	64.7 ± 7.8 c	72.3 ± 13.5 c	445 ± 72 c
	306	11.2 ± 5.0 c	59.8 ± 10.0 c	92.4 ± 14.0 dc	304 ± 33 c
Significance		**	**	*	**

[a] Sugars presented as $\mu g \cdot cm^{-2}$

[b] Starch content presented as μg glucose equivalents $\cdot cm^{-2}$

[c] *, **, and ns represent significant (P = 0.05 and P = 0.01) and not significantly different, respectively (d, c, f indicate significant differences between values, using the Student-Newman-Keuls multiple range test; ns, no significant difference).

Table 11.Carbohydrate content within source leaves of transgenic tobacco plants expressing wild-type TMV-MP (lines 277, 2004), a 10 amino acid C-terminal deletion mutant of the TMV-MP (second, independent transformant, line MP1-1), and the vector controls (lines 306 and 3001). Experimental details as in Table 8 (mean ± SE, n = 5).

Time of day	Plant line	Sucrose[a]	Glucose	Fructose	Starch[b]
13:00	277	194.6 ± 23.7 d	311.1 ± 24.7 d	182.0 ± 18.0	1129 ± 98 d
	MP1-1	106.9 ± 11.6 c	175.4 ± 25.2 c	143.8 ± 16.9	500 ± 15 c
	306	112.1 ± 20.0 c	173.4 ± 32.9 c	143.9 ± 23.5	588 ± 80 c
Significance[c]		**	**	ns	**
	2004	158.1 ± 20.9 f	317.9 ± 25.3 f	182.5 ± 32.1 f	956 ± 141 f
	3001	62.1 ± 8.9 g	69.5 ± 16.7 g	75.3 ± 16.5 g	301 ± 29 g
Significance		**	**	**	**
19:00	277	208.2 ± 21.0 d	277.8 ± 21.3 d	165.3 ± 14.3 d	1438 ± 146 d
	MP1-1	99.3 ± 7.1 c	128.7 ± 12.6 c	104.7 ± 6.9 c	584 ± 103 c
	306	119.7 ± 21.7 c	146.3 ± 28.8 c	111.7 ± 16.8 c	822 ± 123 c
Significance		**	**	*	**
	2004	187.3 ± 19.6 f	271.7 ± 11.0 f	150.2 ± 15.8 f	1091 ± 59 f
	3001	64.8 ± 3.8 g	54.2 ± 8.2 g	74.0 ± 7.2 g	396 ± 39 g
Significance		**	**	**	**
07:00	277	140.3 ± 13.4 d	286.1 ± 13.5 d	172.2 ± 15.6 d	1048 ± 89 d
	MP1-1	46.0 ± 6.0 c	86.7 ± 19.0 c	96.2 ± 11.7 c	424 ± 66 c
	306	49.3 ± 8.5 c	92.0 ± 24.4 c	98.8 ± 15.2 c	488 ± 135 c
Significance		**	**	**	**
	2004	110.6 ± 18.2 f	247.5 ± 31.2 f	154.8 ± 21.9 f	1132 ± 129 f
	3001	38.6 ± 1.2 g	54.3 ± 19.2 g	54.4 ± 6.2 g	189 ± 41 g
Significance		**	**	**	**

[a] Sugars presented as $\mu g \cdot cm^{-2}$

[b] Starch content presented as μg glucose equivalents $\cdot cm^{-2}$

Table 12. KN1 Capacity to Interact with Plasmodesmata Explored Using Mutational Analysis.

KN1	Microinjections[a]	Movement[b]
KN1 wild-type	11 (14)	+
KN1 mutants:		
M2Y39	8 (11)	+
M9Y12	9 (9)	+
M11Y51	3 (40)	-

[a] Number of injections in which KN1 protein moved from target cell into surrounding tissue (total number of injections in each experiment given in parenthesis).

[b] Movement of fluorescently labeled KN1 protein was detected using a fluorescence microscope and permanent images of were recorded on videotape. + = movement out of the target cell; - = no movement out of injected target cell.

Table 13. KN1 has the Ability to Interact with its own mRNA and Traffic it through Plasmodesmata.

Injected material	Microinjections[a] Movement[b]	
Knotted-1 mRNA	0 (15)	-
Knotted-1 mRNA plus KN1	11 (12)	+
Knotted-1 mRNA plus mutant KN1 M11Y51	0 (10)	-

[a] Number of injections in which *Knotted-1* mRNA moved from target cell into surrounding tissue (total number of injections in each experiment given in parenthesis).

[b] Movement of fluorescently labeled *Knotted-1* mRNA was detected using a fluorescence microscope and permanent images of were recorded on videotape. + = movement out of the target cell; - = no movement out of injected target cell.

Table 14. *Growth characteristics and biomass partitioning in vector control tobacco plants (306) versus those in transgenic lines expressing the wild type (277) and N-terminal deletion mutant form (Mn-5) of the TMV-MP grown under low and high light levels.*

Growth light intensity[1] (μmol m^{-2} s^{-1})	Transgenic line	Plant height (cm)	Number of leaves	Mean internodal length (cm)	Dry weight (g)			
					Leaves	Stems	Root	R/S
1200	306	107 ± 7	28 ± 2.0	3.8	13.2 ± 0.8	10.6 ± 0.5	3.7 ± 0.4	0.16 ± 0.006
	277	89 ± 5	26 ± 1.4	3.4	11.4 ± 0.4	7.4 ± 0.8	2.0 ± 0.3	0.11 ± 0.009
	Mn-5	56 ± 4	17 ± 1.5	3.3	7.0 ± 0.6	4.2 ± 0.3	1.2 ± 0.1	0.11 ± 0.006
150	306	124 ± 4	22 ± 0.8	5.6	9.6 ± 0.8	8.1 ± 0.7	1.1 ± 0.2	0.06 ± 0.009
	277	94 ± 6	17 ± 1.5	5.5	5.6 ± 0.8	4.5 ± 0.8	0.6 ± 0.08	0.06 ± 0.007
	Mn-5	56 ± 7	15 ± 1.5	3.7	3.3 ± 0.5	1.8 ± 0.4	0.3 ± 0.06	0.06 ± 0.006

[1]Plants were grown in a greenhouse and the light intensity was adjusted using shade cloth. Measurements of the spectral distribution under the two light regimes indicated that only the actual intensity was altered. Photoperiod was approx. 14h day/10h night.

Table 15. A maize homeodomain protein, KN1, interacts with plasmodesmata to increase size exclusion limit of maize mesophyll cells[a] and potentiates its own cell-to-cell transport.

Injected material	Microinjections	
	(n)	Movement (%)[b]
Lucifer yellow CH	12	11 (92)
FITC-KN1	12	10 (83)
9.4 kDa FITC-dextran	12	1 (8)
KN1 + 9.4 kDa FITC-dextran	11	9 (82)

[a]The normal SEL of plasmodesmata in such plant cells is 800 - 1000 Da (5). The largest material known to pass through mesophyll plasmodesmata is a viral movement protein of 35 kDa (6). Developing maize leaves (one to two cm in width) from young seedlings (14 days post-germination) were used in these experiments.

[b] Number of injections in which the fluorescently labeled probe moved from the injected cell into surrounding tissue. (n) total number of injections in each experiment. Fluorescence was detected using a Leitz Orthoplan epi-illumination microscope coupled with a Hamamatsu model C1966-20 analytical system, and permanent images were recorded on videotape.

Table 16. KN1 interacts with plasmodesmata to increase size exclusion limit of tobacco mesophyll cells and potentiates its own cell-to-cell transport.

Injected material	Microinjections	
	(n)	Movement (%)[a]
Lucifer yellow CH	54	49 (91)
FITC-KN1	33	29 (88)
FITC-labeled bacterial proteins	10	0 (0)
9.4 kDa FITC-dextran	35	3 (9)
KN1 + 9.4 kDa FITC-dextran	38	29 (76)
20 kDa FITC-dextran	11	1 (9)
KN1 + 20 kDa FITC-dextran	19	16 (84)
KN1 + 39 kDa FITC-dextran	25	5 (20)
20 kDa FITC-soybean trypsin inhibitor	15	2 (13)
KN1 + 20 kDa FITC-soybean trypsin inhibitor	10	10 (100)
FITC-KN1 (M6)[b]	15	1 (7)
KN1 (M6) + 9.4 kDa FITC-dextran	16	3 (19)
KN1 (M1) + 9.4 kDa FITC-dextran	11	8 (73)
KN1 (M2) + 9.4 kDa FITC-dextran	8	6 (75)
KN1 (M3) + 9.4 kDa FITC-dextran	8	6 (75)
KN1 (M4) + 9.4 kDa FITC-dextran	12	8 (75)
KN1 (M5) + 9.4 kDa FITC-dextran	9	9 (100)
KN1 (M7) + 9.4 kDa FITC-dextran	7	6 (86)
KN1 (M8) + 9.4 kDa FITC-dextran	8	6 (75)
KN1 (M9) + 9.4 kDa FITC-dextran	16	10 (63)

[a]Number of injections in which the fluorescently labeled probe moved from the injected cell into surrounding tissue. (n) total number of injections in each experiment. Movement of fluorescently labeled *kn1* mRNA, or CMV RNA, was detected as described in Table 1.

[b] Details on the amino acid changes engineered for each KN1 mutant are given in Figure 4.

Table 17. KN1 can selectively traffic its own mRNA through plasmodesmata.

Injected material	Microinjections (n)	Movement (%)[a]
kn1 sense RNA-TOTO	25	1 (4)
kn1 sense RNA-TOTO plus KN1	22	20 (91)
kn1 sense RNA-TOTO plus KN1 M6	10	0 (0)
kn1 antisense RNA-TOTO	10	2 (20)
kn1 antisense RNA-TOTO plus KN1	10	0 (0)
CMV RNA-TOTO plus CMV 3a MP	15	13 (87)
CMV RNA-TOTO plus KN1	15	3 (20)
kn1 sense RNA-TOTO plus CMV 3a MP	15	12 (80)

[a] Number of injections in which *kn1* mRNA, or CMV RNA, moved from target cell into surrounding tissue. (n) number of injections in each experiment. Movement of fluorescently labeled *kn1* mRNA, or CMV RNA, was detected as described in Table 15.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT: Lucas, William J.
(ii) TITLE OF INVENTION: Regulation of Plant Development and Physiology Through Plasmodesmatal Macromolecular Transport of Proteins and Oligonucleotides
(iii) NUMBER OF SEQUENCES: 1
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(F) ZIP: 95608
(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette, 3.5 inch, 1.44 Mb
(B) COMPUTER: IBM compatibles
(C) OPERATING SYSTEM: MS-DOS
(D) SOFTWARE: Word Perfect 6.0 (DOS)
(vi) CURRENT APPLICATION DATA:
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(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO. 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 359 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: protein
(ix) FEATURE:
(D) OTHER INFORMATION: nuclear-localized transcription factor encoded by the maize knotted 1 (kn1) homeobox gene
(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 1:

```
Met Glu Glu Ile Thr Gln His Phe Gly Val Gly Ala Ser Ser His Gly
                5                  10                  15

His Gly His Gly Gln His His His His His His His His Pro Trp
            20                  25                  30

Ala Ser Ser Leu Ser Ala Val Val Ala Pro Leu Pro Pro Gln Pro Pro
        35                  40                  45

Ser Ala Gly Leu Pro Leu Thr Leu Asn Thr Val Ala Ala Thr Gly Asn
    50                  55                  60
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Ser Gly Gly Ser Gly Asn Pro Val Leu Gln Leu Ala Asn Gly Gly Gly
 65                  70                  75                  80

Leu Leu Asp Ala Cys Val Lys Ala Lys Glu Pro Ser Ser Ser Ser Pro
                85                  90                  95

Tyr Ala Gly Asp Val Glu Ala Ile Lys Ala Lys Ile Ile Ser His Pro
            100                 105                 110

His Tyr Tyr Ser Leu Leu Thr Ala Tyr Leu Glu Cys Asn Lys Val Gly
        115                 120                 125

Ala Pro Pro Glu Val Ser Ala Arg Leu Thr Glu Ile Ala Gln Glu
    130                 135                 140

Val Glu Ala Arg Gln Arg Thr Ala Leu Gly Gly Leu Ala Ala Ala Thr
145                 150                 155

Glu Pro Glu Leu Asp Gln Phe Met Glu Ala Tyr His Glu Met Leu Val
160                 165                 170                 175

Lys Phe Arg Glu Glu Leu Thr Arg Pro Leu Gln Glu Ala Met Glu Phe
                180                 185                 190

Met Arg Arg Val Glu Ser Gln Leu Asn Ser Leu Ser Ile Ser Gly Arg
            195                 200                 205

Ser Leu Arg Asn Ile Leu Ser Ser Gly Ser Ser Glu Glu Asp Gln Glu
        210                 215                 220

Gly Ser Gly Gly Glu Thr Glu Leu Pro Glu Val Asp Ala His Gly Val
225                 230                 235

Asp Gln Glu Leu Lys His His Leu Leu Lys Lys Tyr Ser Gly Tyr Leu
240                 245                 250                 255

Ser Ser Leu Lys Gln Glu Leu Ser Lys Lys Lys Lys Lys Gly Lys Leu
                260                 265                 270

Pro Lys Glu Ala Arg Gln Gln Leu Leu Ser Trp Trp Asp Gln His Tyr
            275                 280                 285

Lys Trp Pro Tyr Pro Ser Glu Thr Gln Lys Val Ala Leu Ala Glu Ser
        290                 295                 300

Thr Gly Leu Asp Leu Lys Gln Ile Asn Asn Trp Phe Ile Asn Gln Arg
305                 310                 315

Lys Arg His Trp Lys Pro Ser Glu Glu Met His His Leu Met Met Asp
320                 325                 330                 335

Gly Tyr His Thr Thr Asn Ala Phe Tyr Met Asp Gly His Phe Ile Asn
                340                 345                 350

Asp Gly Gly Leu Tyr Arg Leu Gly
            355                 359
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CLAIMS

1. A molecular mechanism for transporting an oligonucleotide from a first cell of a plant to a nonadjacent second cell of said plant, said cells being in plasmodesmatal communication with one another, the mechanism comprising:

a. a movement protein capable of transport through plasmodesmata;

b. means for causing said oligonucleotide to be transported through plasmodesmata with said movement protein.

2. The mechanism of Claim 1, wherein said oligonucleotide codes for said movement protein.

3. A mechanism for blocking herbivory in higher plants, comprising:

a. an oligonucleotide which codes for a polypeptide inducer of plant defensive genes; and,

b. means for passing said oligonucleotide through a plasmodesma.

4. The mechanism of Claim 3, wherein said oligonucleotide is messenger RNA.

5. The mechanism of Claim 3, wherein said polypeptide inducer of plant defensive genes is systemin.

6. The mechanism of Claim 3, wherein said means for passing said oligonucleotide through a plasmodesma is a movement protein.

7. The mechanism of Claim 6, wherein said movement protein is coded for by said oligonucleotide.

8. A method for studying cell-to-cell macromolecular transport through plasmodesmata, comprising the steps of:

a. labeling a macromolecule with a fluorescent dye;

b. injecting said dye-labeled macromolecule along with a putative plasmodesmatal movement protein into a first cell;

c. examining a second cell in plasmodesmatal communication with said first cell for presence of said dye.

9. A method for controlling biomass partitioning in a plant, comprising the steps of:

a. modifying a gene which codes for a protein able to interact with or traffic cell-to-cell through plasmodesmata; and,

b. causing expression of a protein product from said modified gene.

10. The method of Claim 9, wherein said protein product is unable to interact with or traffic cell-to-cell through plasmodesmata.

11. The method of Claim 9, wherein said gene is a viral gene.

12. The method of Claim 9, wherein said gene is a tobacco mosaic virus gene.

13. The method of Claim 9, wherein said gene codes for a viral movement protein.

14. The method of Claim 9, wherein said gene is a plant gene.

15. The method of Claim 14, wherein said gene codes for a movement protein.

16. The method of Claim 14, wherein said gene is

endogenous to said plant in which biomass partitioning is controlled.

17. A method for controlling carbon metabolism in a plant, comprising the steps of:

a. modifying a gene which codes for a protein able to interact with or traffic cell-to-cell through plasmodesmata; and,

b. causing expression of a protein product from said modified gene.

18. The method of Claim 17, wherein said protein product is unable to interact with or traffic cell-to-cell through plasmodesmata.

19. The method of Claim 17, wherein said gene is a viral gene.

20. The method of Claim 17, wherein said gene is a tobacco mosaic virus gene.

21. The method of Claim 17, wherein said gene codes for a viral movement protein.

22. The method of Claim 17, wherein said gene is a plant gene.

23. The method of Claim 22, wherein said gene codes for a movement protein.

24. The method of Claim 22, wherein said gene is endogenous to said plant in which carbon metabolism is controlled.

25. A method for controlling the overall size of a plant, comprising the steps of:

a. modifying a gene which codes for a protein able

to interact with or traffic cell-to-cell through plasmodesmata; and,

b. causing expression of a protein product from said modified gene.

26. The method of Claim 25, wherein said protein product is unable to interact with or traffic cell-to-cell through plasmodesmata.

27. The method of Claim 25, wherein said gene is a viral gene.

28. The method of Claim 25, wherein said gene is a tobacco mosaic virus gene.

29. The method of Claim 25, wherein said gene codes for a viral movement protein.

30. The method of Claim 25, wherein said gene is a plant gene.

31. The method of Claim 30, wherein said gene codes for a movement protein.

32. The method of Claim 30, wherein said gene is endogenous to said plant in which overall size is controlled.

33. A method for interfering with a plant's endogenous signal transduction pathway, comprising the steps of:

a. modifying a gene which codes for a protein able to interact with or traffic cell-to-cell through plasmodesmata; and,

b. causing expression of a protein product from said modified gene.

34. The method of Claim 33, wherein said protein product is unable to interact with or traffic cell-to-cell

through plasmodesmata.

35. The method of Claim 33, wherein said gene is a viral gene.

36. The method of Claim 33, wherein said gene is a tobacco mosaic virus gene.

37. The method of Claim 33, wherein said gene codes for a viral movement protein.

38. The method of Claim 33, wherein said gene is a plant gene.

39. The method of Claim 38, wherein said gene codes for a movement protein.

40. The method of Claim 38, wherein said gene interfering with said plant's endogenous signal transduction pathway is endogenous to said plant.

41. The method of Claim 33, wherein said gene is the *Knotted-1* gene.

42. The method of Claim 33, wherein said protein product is a modified form of the KNOTTED protein.

43. A method of inducing cell-to-cell transmission of a plant-encoded protein, comprising the steps of:

a. introducing into a target cell of an intact plant an oligonucleotide coding for said plant-encoded protein, and also coding for a protein able to transport said oligonucleotide through a plasmodesma; and,

b. causing expression of a protein product from said oligonucleotide.

44. The method of Claim 43, where said introduction is

effected by microinjection.

45. A method for altering a plant's height and weight, without altering said plant's mean internodal length, comprising the steps of:

a. introducing into said plant a gene which codes for a protein able to interact with or traffic cell-to-cell through plasmodesmata;

b. causing expression of a protein product from said modified gene; and,

c. exposing said plant to high light conditions.

46. The method of Claim 45, wherein said gene is a viral gene, further including the step of modifying said gene before introducing said gene into said plant.

47. The method of Claim 45, wherein said protein product of said gene is unable to interact with or traffic cell-to-cell through plasmodesmata.

48. The method of Claim 45, wherein said light conditions comprise approximately 1200 μmol m^{-2} s^{-1} of irradiance.

49. A method for altering a plant's mean internodal length without altering said plant's height, comprising the steps of:

a. introducing into said plant a gene which codes for a protein able to interact with or traffic cell-to-cell through plasmodesmata;

b. causing expression of a protein product from said modified gene; and,

c. exposing said plant to low light conditions.

50. The method of Claim 49, wherein said light conditions comprise approximately 150 μmol m^{-2} s^{-1} of irradiance.

51. The mechanism of Claim 1, wherein said first cell resides in a first cell layer, and wherein said second cell resides in a second cell layer.

52. The mechanism of Claim 51, wherein said first and second cell layers are meristematic tissue cell layers.

53. The mechanism of Claim 1, wherein said first cell resides in a first tissue, and wherein said second cell resides in a second tissue.

54. The mechanism of Claim 53, wherein said first tissue is mesophyll and wherein second tissue is vascular tissue.

55. The mechanism of Claim 53, wherein said first tissue is epidermal tissue and wherein second tissue is vascular tissue.

56. The mechanism of Claim 53, wherein said first tissue is vascular tissue and wherein second tissue is mesophyll.

57. A method for controlling cell fate of a first cell in a first tissue in a plant, comprising the steps of:

a. modifying a gene which codes for a protein able to interact with or traffic cell-to-cell through plasmodesmata; and,

b. causing expression of a protein product from said modified gene in a second cell in a second tissue.

58. The method of Claim 57, wherein said protein product is able to control development in said first cell.

59. The method of Claim 57, wherein said protein product is able to control differentiation in said first cell.

60. The method of Claim 57, wherein said first tissue is a single layer of meristematic tissue.

61. The method of Claim 57, wherein said first tissue is an outer meristematic tissue layer.

62. The method of Claim 57, wherein said first tissue is an inner meristematic tissue layer.

63. The method of Claim 57, wherein said second tissue is a single layer of meristematic tissue.

64. The method of Claim 57, wherein said second tissue is an outer meristematic tissue layer.

65. The method of Claim 57, wherein said second tissue is an inner meristematic tissue layer.

66. The method of Claim 57, wherein said first tissue is mesophyll and wherein second tissue is vascular tissue.

67. The method of Claim 66, wherein said protein product is able to control differentiation in said mesophyll.

68. The method of Claim 57, wherein said first tissue is epidermal tissue and wherein second tissue is vascular tissue.

69. The method of Claim 68, wherein said protein product is able to control differentiation in said epidermal tissue.

70. The method of Claim 57, wherein said first tissue is vascular tissue and wherein second tissue is mesophyll.

71. The method of Claim 70, wherein said protein product is able to control differentiation in said vascular

tissue.

72. A method for affecting cell fate in a first cell in a first cell layer, including the steps of:

a. inducing the presence of mRNA in a second cell disposed in a second cell layer directly adjacent to said first cell layer, wherein said mRNA codes for a protein able to affect cell fate in said first cell, and wherein said protein is also able to be selectively transported via plasmodesmata between cells in said first layer and cells in said second layer;

b. causing expression of said protein from said mRNA in said second cell layer.

73. A protein able to interact with or traffic cell-to-cell through plasmodesmata comprising the amino acid sequence of SEQ ID NO:1.

74. A protein able to interact with or traffic cell-to-cell through plasmodesmata comprising the amino acid sequence of 3 amino acids, running in the N-terminal to C-terminal direction, from amino acid position 242 to amino acid position 244, inclusive, in SEQ ID NO:1.

75. A modified form of a protein which, in its wild type form, is able to interact with or traffic cell-to-cell through plasmodesmata, comprising: the amino acid sequence of SEQ ID NO:1 modified by deletion of a plurality of adjacent histines.

76. The protein of Claim 75, wherein an amino acid sequence of 9 histines is deleted, running in the N-terminal to C-terminal direction, from amino acid position 22 to amino acid position 30, inclusive, in SEQ ID NO:1.

77. A modified form of a protein which, in its wild type form, is able to interact with or traffic cell-to-cell

through plasmodesmata, comprising: the amino acid sequence of SEQ ID NO:1 modified by replacement of a plurality of amino acids with alanines.

78. The protein of Claim 77, modified by replacement of the amino acid sequence of 2 amino acids, running in the N-terminal to C-terminal direction, from amino acid position 25 to amino acid position 26, inclusive, in SEQ ID NO:1.

79. The protein of Claim 77, modified by replacement of the amino acid in position 162 and the amino acid in position 164, running in the N-terminal to C-terminal direction, in SEQ ID NO:1.

80. The protein of Claim 77, modified by replacement of the amino acid sequence of 3 amino acids, running in the N-terminal to C-terminal direction, from amino acid position 219 to amino acid position 221, inclusive, in SEQ ID NO:1.

81. The protein of Claim 77, modified by replacement of the amino acid in position 242 and the amino acid in position 244, running in the N-terminal to C-terminal direction, in SEQ ID NO:1.

82. The protein of Claim 77, modified by replacement of the amino acid sequence of 3 amino acids, running in the N-terminal to C-terminal direction, from amino acid position 265 to amino acid position 267, inclusive, in SEQ ID NO:1.

83. The protein of Claim 77, modified by replacement of the amino acid sequence of 2 amino acids, running in the N-terminal to C-terminal direction, from amino acid position 273 to amino acid position 274, inclusive, in SEQ ID NO:1.

84. The protein of Claim 77, modified by replacement of the amino acid sequence of 2 amino acids, running in the N-terminal to C-terminal direction, from amino acid position

320 to amino acid position 321, inclusive, in SEQ ID NO:1.

85. The protein of Claim 77, modified by replacement of the amino acid sequence of 2 amino acids running in the N-terminal to C-terminal direction from amino acid position 327 to amino acid position 328, inclusive, and, by replacement of the amino acid sequence of 2 amino acids running from amino acid position 330 to amino acid position 331, inclusive, in SEQ ID NO:1.

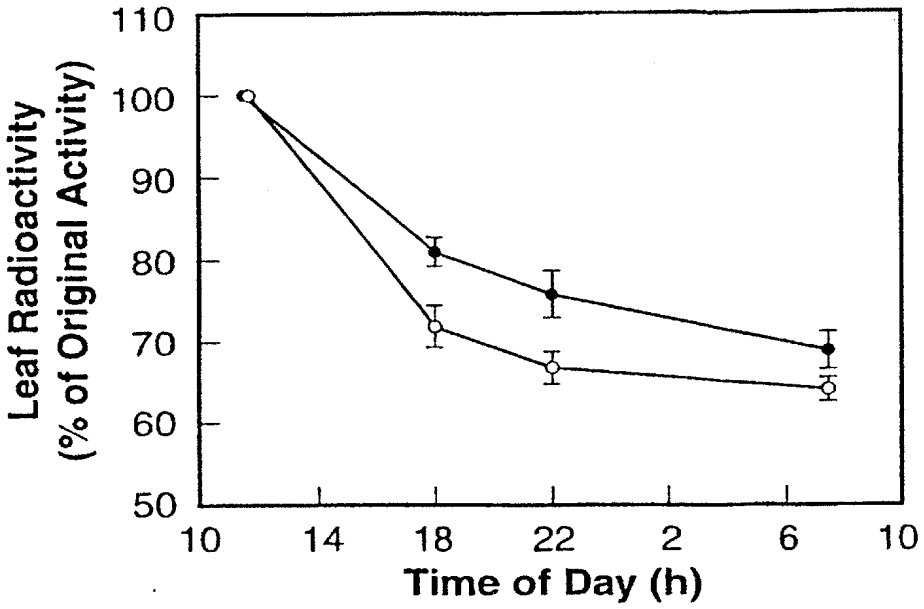

FIG. – 1A

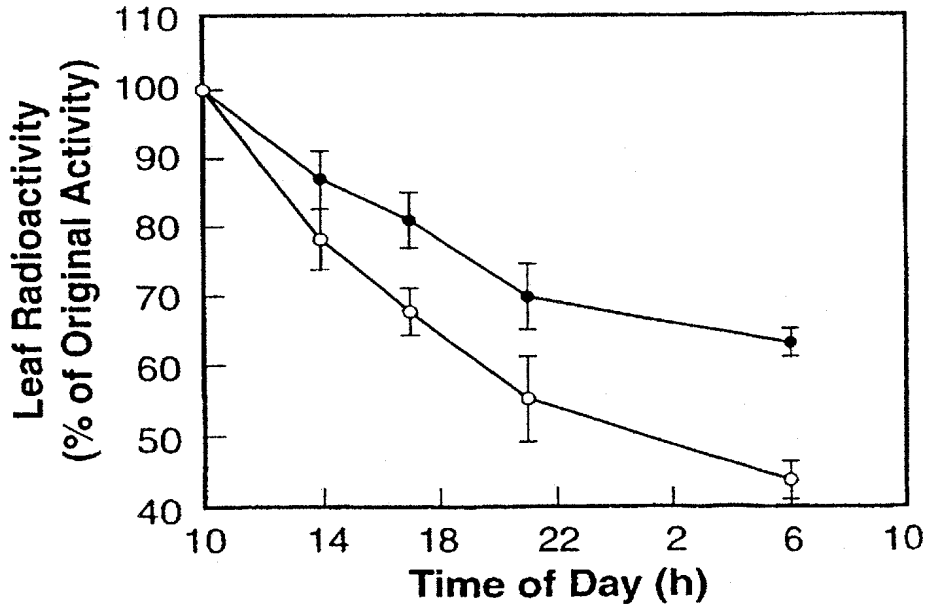

FIG. – 1B

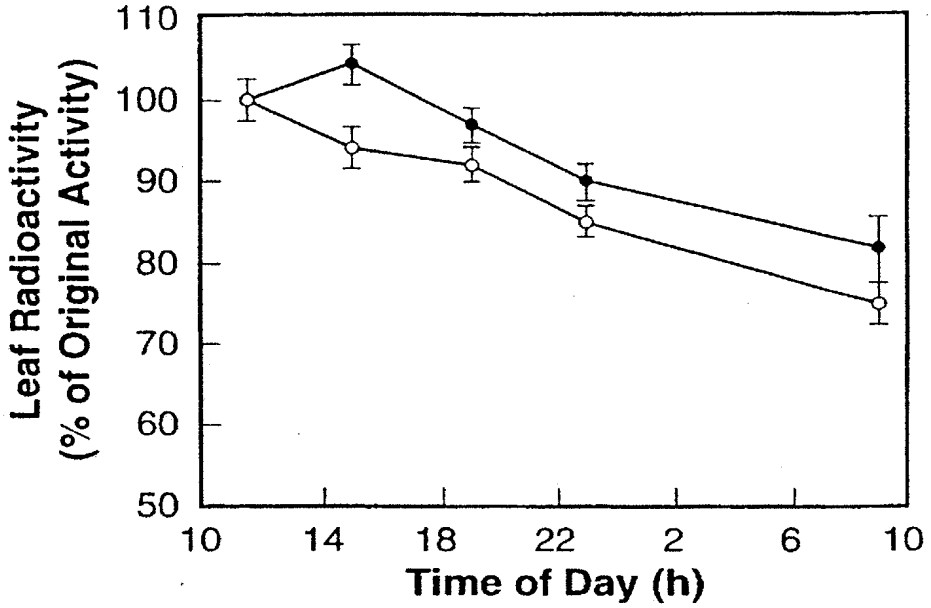

FIG. – 1C

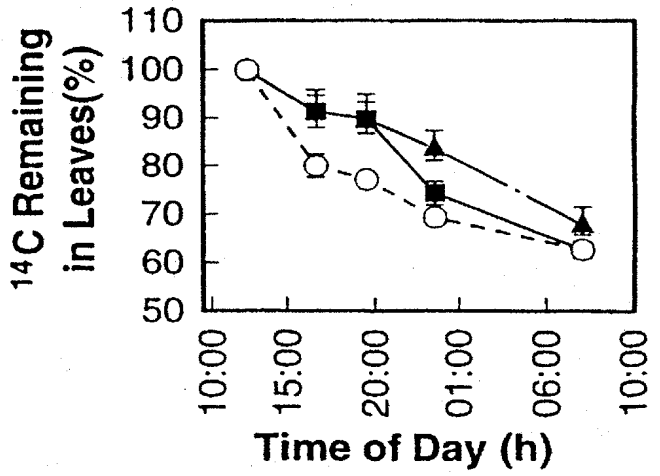

FIG. – 2A

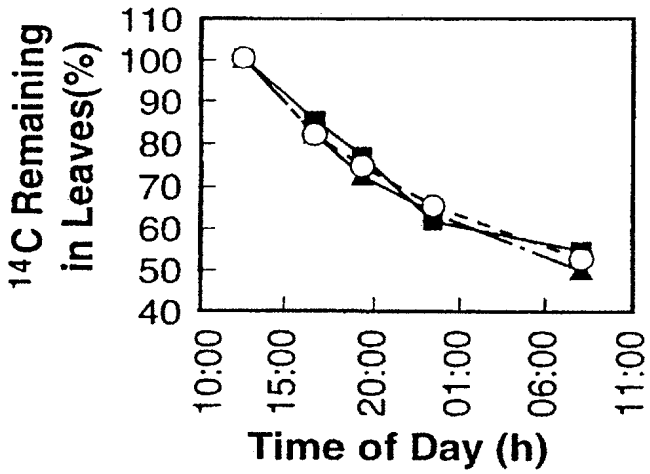

FIG. – 2B

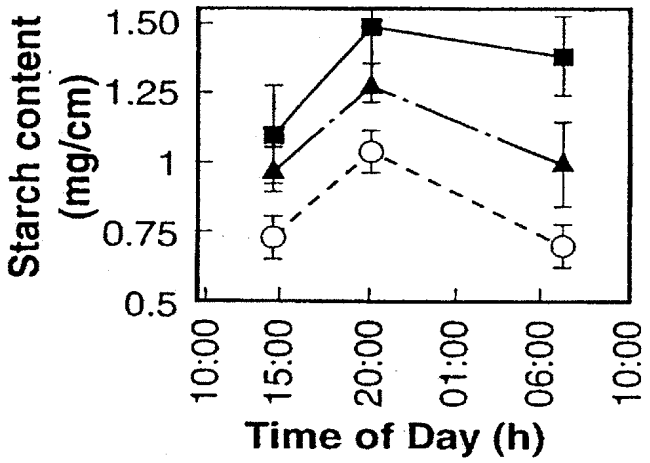

FIG. – 2C

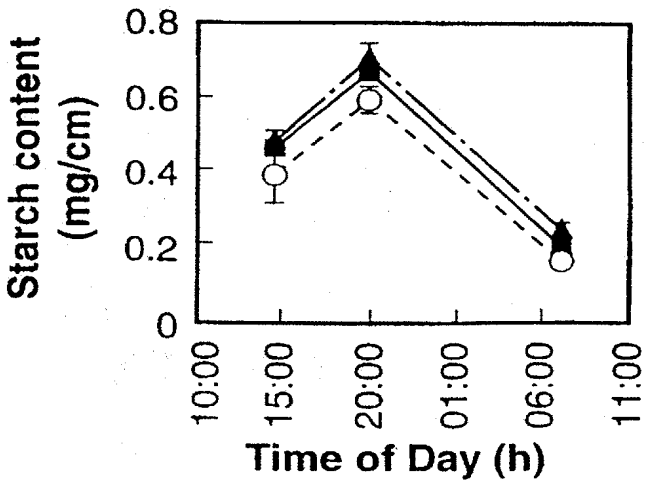

FIG. – 2D

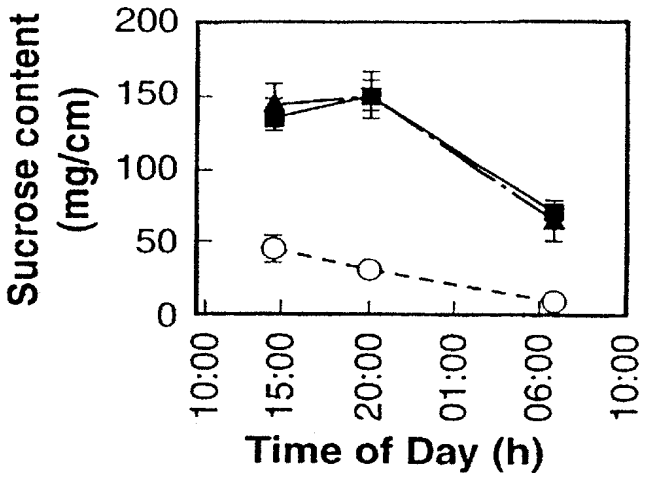

FIG. – 2E

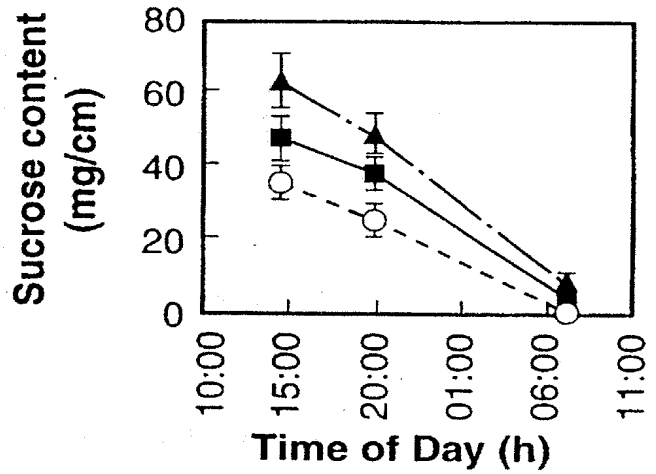

FIG. – 2F

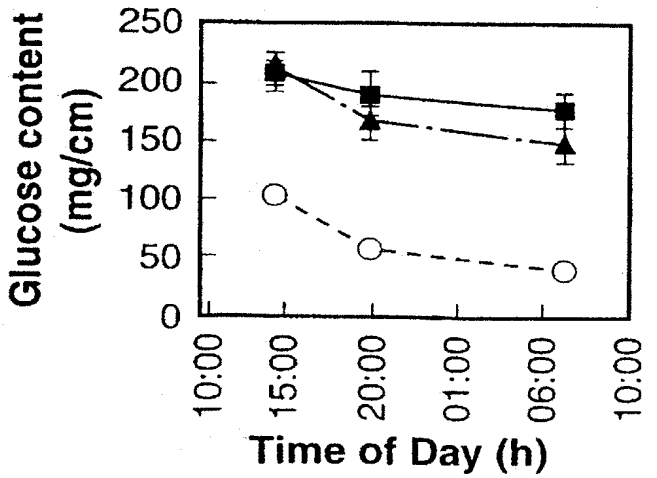

FIG. – 2G

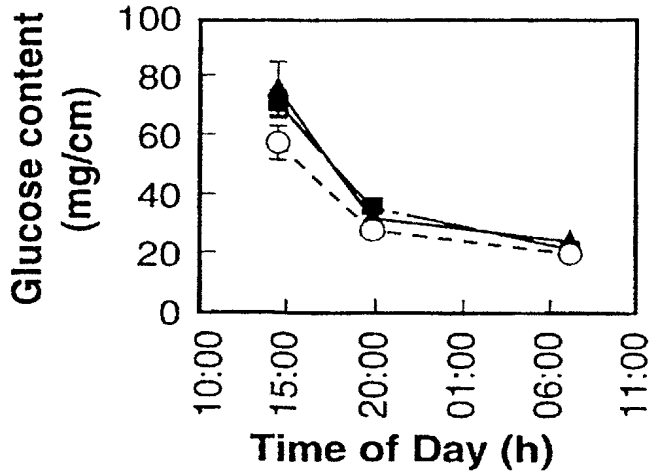

FIG. – 2H

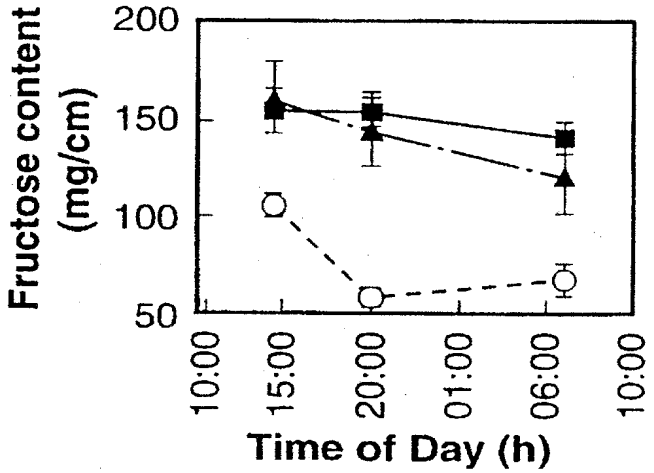

FIG. – 2I

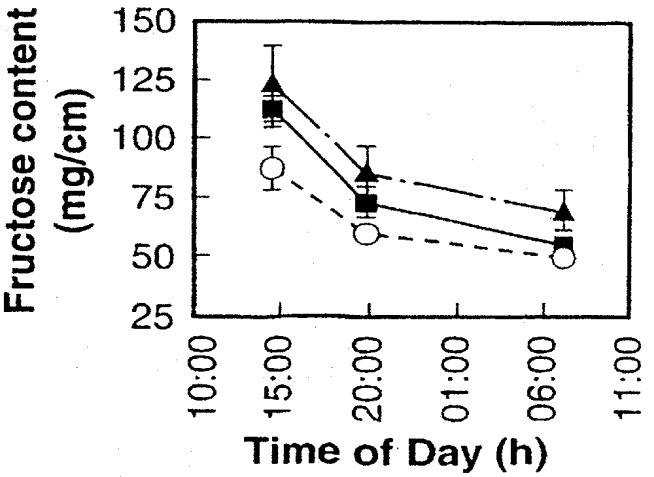

FIG. – 2J

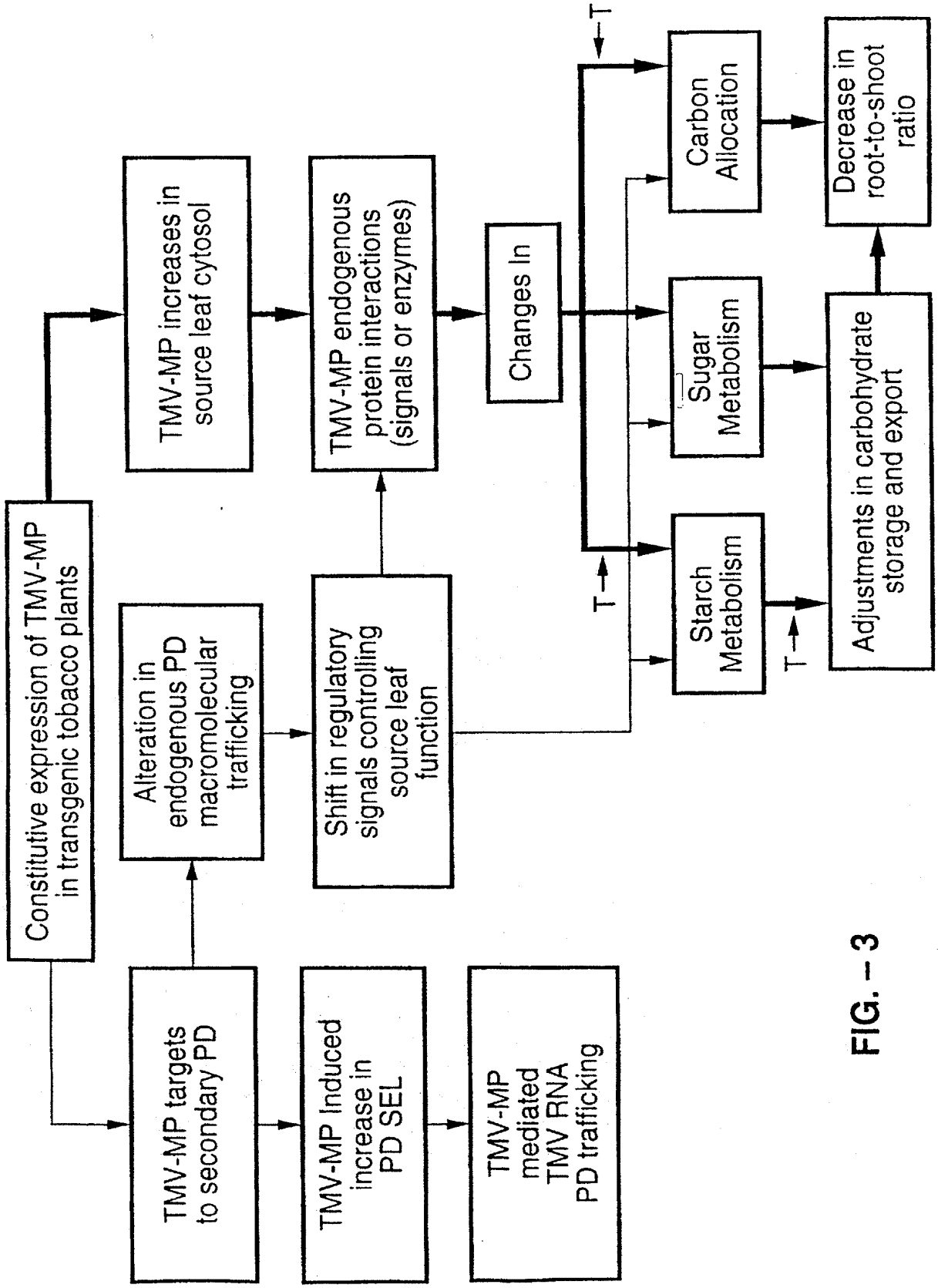

FIG. – 3

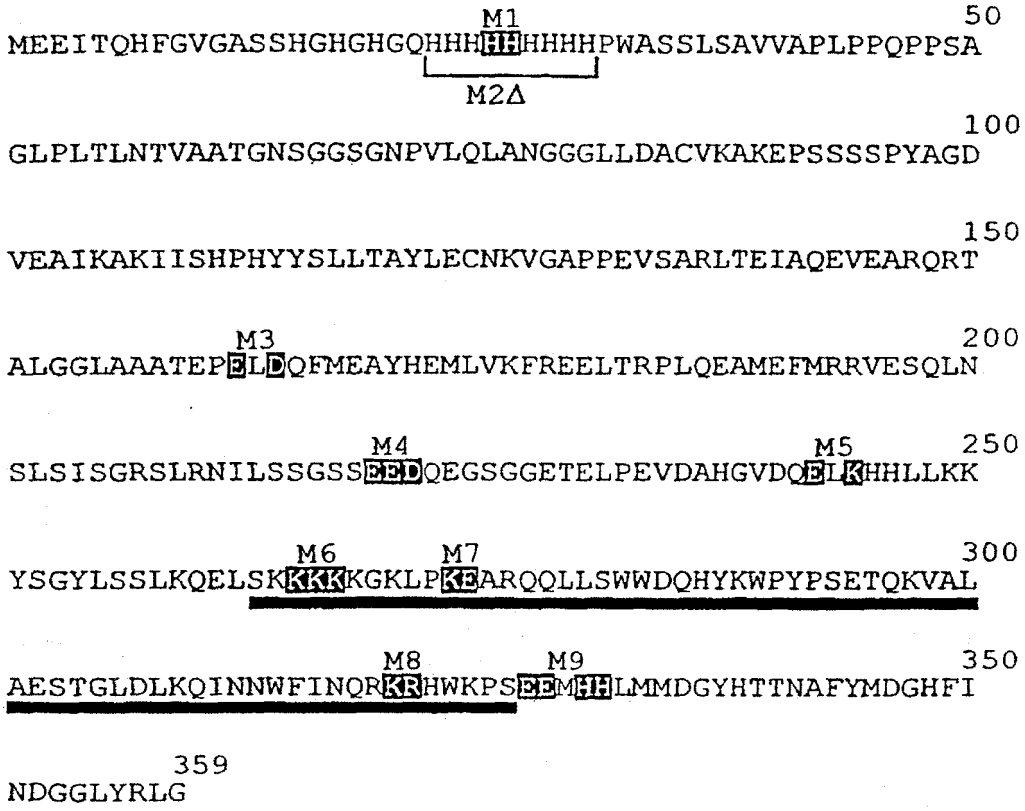

Fig. 4. KN1 alanine scanning and deletion mutants generated to identify protein domain(s) essential for KN1-plasmodesmal interaction. (Conventional single-letter amino acid codes are used here for simplicity. See SEQ ID NO:1 for corresponding three-letter amino acid codes.) Amino acid residues changed to alanines are marked with black boxes, with the assigned number of each mutant indicated above the site. Deletion mutant M2 was generated by the removal of a 9 histidine stretch from position 22 to 30. The residues associated with the KN1 homeodomain are underlined. The domains affected by these mutations are as follows: M1 and M2, histidine-rich region of unknown function; M3 and M4, regions conserved between certain *kn1* related genes; M5, the "ELK" region which is conserved in all KN1-like homeodomain proteins; M6, potential nuclear localization sequence; M7, homeodomain 1st helix; M8, homeodomain 3rd helix; M9, C-terminal border of homeodomain.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/19260

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A10H 5/00; C12N 5/04, 15/29, 15/40, 15/82
US CL :435/69.1, 172.3, 240.4; 536/23.6, 23.72; 800/205
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.3, 240.4; 536/23.6, 23.72; 800/205

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	FUJIWARA et al. Cell-to-Cell Trafficking of Macromolecules through Plasmodesmata Potentiated by the Red Clover Necrotic Mosaic Virus Movement Protein. The Plant Cell, December 1993, Vol. 5, No. 12, pages 1783-1794, especially page 1783.	1-2 ---------- 3-7,43,44,51-56,72
X -- Y	DING et al. Secondary Plasmodesmata Are Specific Sites of Localization of the Tobacco Mosaic Virus Movement Protein in Transgenic Tobacco Plants. The Plant Cell. August 1992, Vol. 4, No. 8, pages 915-928, especially pages 915 and 919.	1,2,51,53-56 ---------- 3 7,43,44,52,72

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 08 MARCH 1997	Date of mailing of the international search report 21 MAR 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAVID T. FOX Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/19260

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	TAKAMATSU et al. Production of enkephalin in tobacco protoplasts using tobacco mosaic virus RNA vector. FEBS Letters. August 1990, Vol. 269, No. 1, pages 73-76, especially page 74.	1-2 --- 3-7,43,44,51-56,72
Y	HILDER et al. A novel mechanism of insect resistance engineered into tobacco. Letters to Nature. 12 November 1987, Vol. 300, pages 160-163, especially pages 160-161.	3-7
Y	McGURL et al. Structure, Expression, and Antisense Inhibition of the Systemin Precursor Gene. Science. 20 March 1992, Vol. 255, pages 1570-1573, especially page 1570.	3-7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/19260

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-7, 43-44, 51-56 and 72

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/19260

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-7, 43-44, 51-56 and 72, drawn to a molecular means for protein transport comprising a first gene encoding a plasmodesmata movement protein and a second gene encoding a second protein, and a method for its use comprising transforming plants with the genes and obtaining intercellular movement of desired gene products.

Group II, claim 8, drawn to a method of studying intercellular transport via assaying the movement of a macromolecule labelled with a fluorescent dye, and a putative plasmodesmata movement protein.

Group III, claim(s) 9-42, 45-50 and 57-71, drawn to methods of modifying genes encoding plasmodesmata movement proteins, and expressing the modified genes in transformed plants.

Group IV, claim(s) 73-74, drawn to isolated native plasmodesmata movement proteins.

Group V, claim(s) 75-85, drawn to isolated modified plasmodesmata movement proteins.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The claims are not drawn to a single gene encoding a single plasmodesmata movement protein, but are drawn to a multitude of gene and amino acid sequences, or to any gene encoding any plasmodesmata movement protein. Furthermore, the groups are drawn to physiologically and biochemically divergent processes and products not required by each other.

The invention of Group I involves genes encoding native plasmodesmata movement proteins not required by any other group, and plant transformation and tissue culture techniques not required by Groups II, IV and V.

The invention of Group II, drawn to a second process of use, involves fluorescent dye assays, as well as a multitude of non-proteinaceous macromolecules and putative plasmodesmata movement proteins not required by any other group.

The invention of Group III, drawn to a third process of use, involves methods of modifying genes encoding plasmodesmata movement proteins not required by any of the other groups, and plant transformation and tissue culture techniques not required by Groups II, IV and V.

The invention of Group IV, drawn to a second product, comprises isolated native plasmodesmata movement proteins not required by any of the other groups.

The invention of Group V, drawn to a third product, comprises isolated modified plasmodesmata movement proteins not required by any of the other groups.

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

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(51) International Patent Classification 6 :
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A1

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60/015,051	9 April 1996 (09.04.96)	US
Not furnished	9 August 1996 (09.08.96)	US

(81) Designated States: AL, AU, BB, BG, BR, CA, CN, CZ, EE, GE, HU, IL, IS, JP, KP, KR, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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(74) Agents: LLOYD, Jeff et al.; Saliwanchik & Saliwanchik, Suite A-1, 2421 N.W. 41st Street, Gainesville, FL 32606-6669 (US).

(54) Title: METHODS AND MATERIALS FOR PRODUCING PATHOGEN-RESISTANT PLANTS

(57) Abstract

This invention comprises a mutant plant virus gene which confers resistance on tobacco and tomato plants against tobacco mosaic tobamovirus and tomato mottle geminivirus infections and infection by other related geminiviruses. A gene was initially isolated from the known BC1 gene, between nucleotides 1278 and 2311 of the B component of tomato mottle geminivirus. Upon subcloning of this DNA fragment into an appropriate expression vector and transformation of the gene into tobacco plants, a truncated gene product was produced which confers resistance against viral infection to the recombinant plant in which it is expressed.

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DESCRIPTION

METHODS AND MATERIALS FOR PRODUCING PATHOGEN-RESISTANT PLANTS

Cross Reference to Related Application

This application claims the benefit of U.S. Provisional Application No. 60/015,051, filed April 9, 1996, and U.S. Provisional Application No. 60/002,158, filed August 11, 1995.

Acknowledgement of Government Support

This invention was made with government support under USDA/DSRS CBAG Special Grants Program, grant Nos. 93-34135-8607; 92-34135-7456; and the Florida Tomato Committee Grant No. 90153-C. The government has certain rights in this invention.

Background of the Invention

(i). Field of the Invention

This invention pertains to the field of conferring pathogen resistance to plants. More particularly, the invention is directed to virus-resistant transgenic plants.

(ii). Background of the Invention

Tomato producers suffer significant losses due to tomato mottle geminivirus infection. Currently farmers must purchase chemicals in order to control tomato mottle virus in their tomato fields. Similarly, losses are experienced by farmers producing tobacco as a result of tobacco crop infection by tobacco mosaic tobamoviruses. Accordingly, there is a need for a solution to this problem which is less costly and less damaging to the environment than the chemical controls currently employed.

Production of transgenic plants with enhanced phenotypic characteristics is a relatively recent development in the arsenal available to farmers. Nevertheless, the value of this technology has been demonstrated repeatedly in recent years. However, what is required is the identification of appropriate genes to confer the desired phenotype, in this case, pathogen resistance.

Transformation of plants with portions of viral genomes may result in plants with virus resistance (Beachy, 1993). This phenomenon is known as "pathogen-derived resistance" (Sanford

and Johnson, 1985). The level of resistance obtained is variable. This variability has been attributed to the random nature of the transformation process (Lomonossoff, 1995). Independent lines of plants generated from a single transformation experiment may contain different transgene copy numbers inserted in various chromosomes. Phenotypic differences have been noted among plant lines containing a single copy of the transgene. Some of the variability in transgene expression has also been attributed to tissue culture-induced changes (Phillips *et al.*, 1994). This variability in the phenotype is also observed in subsequent progeny derived from the R_0 plants.

Introduction of a mutation (defective) in one motif of a multimotif protein has been proposed as a strategy for interfering with viral replication. This interference with the function of wild-type genes has been referred to as a dominant negative mutation. Maxwell and his co-workers have constructed transgenic plants expressing a modified tomato mottle geminivirus replication-associated protein (RAP), mutated in a NTP-binding motif, which appears to interfere with viral replication (Hanson, *et al.*, 1991). This dominant negative mutant for the tomato mottle geminivirus RAP gene has been tested for tomato mottle geminivirus resistance in tomatoes. Noris *et al.* (1994, First International Symposium on Geminiviruses, Almeria, Spain) found inhibition of Tomato Yellow Leaf Curl Virus (TYLCV) DNA replication in tobacco protoplasts co-transfected with TYLCV and a construct of a truncated RAP expressed under control of a CaMV 35S promoter.

This control strategy is likely to be very virus specific since the RAP binding sites essential for function have been shown to require a sequence-specific interaction between RAP and the origin of replication (Fontes *et al.*, 1994). This enables the transacting factors of RAP to discriminate between the replication origins of closely related geminiviruses. Because of Geminivirus diversity and adaptability, virus-specific control strategies are of limited value under field conditions.

There have been several reports in recent years relating to the inhibition of infection of certain plants by specific viral pathogens. For example, Von Arnim and Stanley (1992) reported on the inhibition of systemic infection by African Casava Mosaic Virus (ACMV) by a movement protein from the related Geminivirus, Tomato Golden Mosaic Virus (TGMV). This was accomplished by replacing the ACMV coat protein coding sequence with the BL1 or BR1 movement gene sequences from TGMV and then testing the ability of the recombinant ACMV to infect its host, *Nicotiana benthamiana* (which is also the host of TGMV). The authors found that the TGMV gene did not complement the ACMV recombinant, and hypothesized that direct genomic expression of a dominant negative mutant might produce plants resistant against geminiviruses.

Cooper *et al.* (1995) disclosed that transgenic tobacco plants expressing a defective tobacco mosaic virus (TMV) movement protein were resistant to infection by multiple viruses, while

transgenic plants expressing the natural movement protein had increased susceptibility to infection by TMV and other viruses.

Nejidat and Beachy (1990) disclosed that transgenic tobacco plants expressing a TMV coat protein have increased resistance against several of the tobamoviruses. Gilbertson *et al.* (1993) disclosed the reduced pathogenicity of pseudorecombinants of two bipartite geminiviruses, tomato mottle (ToMoV) and TGV-MX1.

Brief Summary of the Invention

We have discovered a mutated plant virus gene which protects tobacco plants against tomato mottle geminivirus and tobacco mosaic tobamovirus infections. This resistance gene has been introduced into tobacco chromosomal DNA by genetic engineering. The transgenic tobacco plants expressing this gene show resistance to tomato mottle geminivirus and tobacco mosaic tobamovirus infections (lack of or reduction of disease symptoms when inoculated with the viruses). The mutated gene can be introduced into chromosomes of desirable tomato and tobacco lines to develop commercially improved tomato and tobacco cultivars/hybrids.

Accordingly, this invention comprises a mutant plant virus gene which confers resistance on tobacco and tomato plants against tobacco mosaic tobamovirus and tomato mottle geminivirus infections, as well as resistance to infections of other related geminiviruses. The known BC1 gene, between nucleotides 1278 and 2311 of the B component of tomato mottle geminivirus, was subcloned into an appropriate expression vector and transformed into tobacco plants. A mutated gene product was produced which confers resistance against viral infection to the recombinant plant in which it is expressed.

One object of this invention is to provide a method for conferring viral resistance on a plant.

Another object of this invention is to provide a mutated BC1 gene and any fragment thereof which confers viral resistance on a plant.

Another object of this invention is to provide novel transgenic plants with enhanced viral resistance.

Other objects and advantages of this invention will become apparent from a review of the complete invention disclosure and the appended claims.

Brief Description of the Figures

Figure 1 is the sequence of the single stranded mutated tomato mottle geminivirus BC1 gene except for positions 1742-1766 which initially were not identified; wild-type nucleotides which are different in the mutant gene are shown in lower case text above the mutant gene sequence.

Figure 2 is the sequence shown in Figure 1 along with its complementary strand; the translational start and stop codons are underlined; the termini are HindIII restriction sites.

Figure 3 is the deduced amino acid sequence of the mutated gene product encoded by the nucleotide sequence of Figure 1, except for positions 151-159 which in initial sequencing efforts were not identified.

Figure 4 shows a comparison of the mutant and wild-type gene products (the mutant protein is the lower sequence).

Figure 5 shows phenotypic comparison of transgenic R_1 tobacco plants expressing BC1 protein of TMoV. Transgenic plants were derived from a R_0 plant which contained two copies of BC1 gene (see Fig. 6) and which did not show any stunting. (A) Plants from left to right: a. transgenic plant (BC1-3-11-5) expressing symptomatic BC1 protein, showing stunting, mottling, and curling on the leaves. Symptoms are more sever than those induced by TMoV infection; b. transgenic plant (BC1-3-11-2) which contains one copy of the non-symptomatic BC1 and the symptomatic BC1 transgene, showing mottling with no stunting; c. transgenic plant (BC1-3-11-6) which contains one copy of non-symptomatic BC1 transgene; and d. non-transgenic tobacco. (B) Plant on the left as in b, Fig. 5A and on the right as in c, Fig. 5A. The plants in A were photographed 45 days after transplanting, and in B 90 days.

Figure 6 shows Southern blot analysis of the R_1 transgenic plant with different phenotypes. Segregation of the BC1 transgene in R_1 generation of transgenic tobacco plants which displayed different phenotypes in Fig. 5 (BC1-3-11-1 and -2, mottling only, -4 and -5, severe stunting and mottling, -6 and -7, no visible symptoms). Blots from BC1-3-16-2 showing stunting and mottling, and BC1-3-6-3 and -4, no visible symptoms are shown for comparative purposes; NT-nontransformed plant; and pKYsBC1, vector construct used for transformation. Genomic DNA of the transgenic plants was extracted and digested with XbaI. Southern blots were subjected to hybridization with ^{32}P-labeled BC1 DNA fragment.

Figure 7 shows Western blot analysis of the P30 fraction of tissue extracts from transgenic R_1 tobacco plants expressing the BC1 gene. Lanes represent extracts from plants described in Fig. 6 except for TMoV-infect. extract from TMoV infected tissue). The subcellular fractions, P1, P30 and S30 were prepared (Pascal *et al.*, 1993) and subjected to SDS-PAGE (Schagger) with some modification and immunoblots using the polyclonal antiserum against expressed BC1 protein. The results of the P1 and S30 fractions are not shown here.

Figure 8 shows Northern blot analysis of transgenic plants which express the BC1 gene, probed with labeled-BC1 DNA. Two BC1 related transcripts were found in the transgenic plants which expressed the full-length BC1 gene, while only one transcript was found in the transgenic

plant which expressed a 3'-truncated form of the BC1 gene (BC1-3-11-6). The samples indicated are as in Fig. 6.

Figures 9A-1 thru 9A-5 and 9B show nucleotide sequences (A) and predicted amino acid sequences (B) of the TMoV BC1 and its transgene mutants. The nucleotide sequence of TMoV BC1 gene from GenBank Accession U14461. The sequence of the PCR amplified BC1 ORF was verified before and after cloning into pGEM-T vector. BC1A sequence determined from an asymptomatic, multicopy transgenic plant which expressed full length BC1 protein. The sequence was analyzed from the PCR product derived from genomic DNA (BC1-3-6-3A). BC1At/r sequence determined from the cDNA, the RT-PCR products, amplified from the total RNA (BC1-3-11-6A). The sequence was also verified by sequencing the PCR product from the genomic DNA and from cloned PCR product. BC1S sequence determined from a symptomatic transgenic plant which expressed full length BC1 protein. The sequence was analyzed after RT-PCR of total RNA (BC1-3-11-5S), after PCR amplification of genomic DNA (BC1-3-11-5S) and after PCR amplification from 3 different lines with a similar phenotype. Note that identical nucleotides and amino acid residues are indicated by (.).

Detailed Description of the Invention

The subject invention concerns a mutated plant virus gene that when expressed in a plant confers on that plant a resistance to infection from plant pathogens. In one embodiment, the mutated virus gene is a BC1 gene of geminivirus. The mutated gene of the present invention can be prepared by inserting the wild-type gene into the genome of a plant and identifying those plants transformed with the gene that exhibit increased resistance to viral infection.

The subject invention also concerns a method for conferring resistance on a plant to infection by plant pathogens. The subject method comprises inserting a wild-type viral movement gene, such as BC1, into the genome of a plant and then identifying those plants that do not exhibit pathogenic symptoms when the inserted gene is expressed but which have enhanced resistance to infection by pathogens.

The subject invention also concerns transgenic plants and plant tissue having a mutated gene of the present invention incorporated into their genome.

The following is a specific example of the subject invention, a method for creating a virus-resistant plant, using the BC1 gene of tomato mottle geminivirus to illustrate the invention. The method is generally and broadly applicable to other plant viruses.

The complete sequence of the BC1 gene of tomato mottle geminivirus is known (Abouzid *et al.*, 1992, herein incorporated by reference). The BC1 gene of tomato mottle geminivirus of the

B component of the genome is isolated in sufficient quantity for subcloning in an expression vector. This may be accomplished by any of several methods well-known in the art. A simple method is to use a pair of specific primers to amplify the desired segment according to the well known polymerase chain reaction (PCR) technique. For this purpose, a useful primer pair such as:

5'-CCCAAGCTTCGAGTTCGAAACTGC-3' (SEQ ID NO. 1) and

5'-CCCAAGCTTAACGAAGTGTGTTTGAC-3' (SEQ ID NO. 2)

may be used. All or portions of the BC1 gene may be used for this purpose.

Once sufficient quantities of the gene are obtained, the gene is cloned into a vector for production of a stable source for mass production of the gene. Any vector known in the art can be used for this purpose, and mass quantities of the vector may be cultured, for example, by transformation of competent bacterial cells such as *E. coli* followed by harvesting of the plasmid DNA. Preferably, the gene is inserted into the multiple cloning site of a vector, such as the commercially available pUC vectors or the pGEM vectors, which allow for excision of the gene having restriction termini adapted for insertion into any desirable plant expression or integration vector. For this purpose any vector in which a strong promoter, such as a viral gene promoter, is operatively linked to the coding sequence of the mutant gene of this invention could be used. For example, the powerful 35S promoter of cauliflower mosaic virus could be used for this purpose. In one embodiment of this invention, this promoter is duplicated in a vector known in the art as pKYLX 71:35S^2 (Morgan *et al.*, 1990). However, other plant expression vectors could be used for this purpose.

Once the gene is excised and re-subcloned into a desirable expression vector, the gene is transformed into a bacterium or other vector which is able to introduce the gene into a plant cell. Alternatively, the gene may be introduced into plant cells by a biolistic method (Carrer, 1995). Preferably, competent *Agrobacterium* cells are used for this purpose, and plant sections are exposed to the *Agrobacterium* harboring the BC1 gene. Regeneration of the plant cells in a selective medium to ensure the efficient uptake of the gene is preferred, following which the regenerated plants are grown under optimized conditions for survival.

As a result of this process, it has been discovered that a large proportion of regenerated tobacco plants which were transgenic for the BC1 gene had a spontaneously mutated gene which expressed a mutated gene product. Unexpectedly, the plants harboring the mutated gene had increased resistance to viral infection by both DNA and RNA plant viruses, without any observed deleterious effects resulting from expression of the mutated BC1 gene (in contrast, expression of the wild-type gene produces disease symptoms).

While those skilled in molecular biology are able to clone the known BC1 gene into a plant expression vector to obtain the mutated gene of the present invention, the mutant gene of this invention has also been deposited prior to filing the instant patent application with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 USA. The mutant gene was cloned in a bacterial vector (pGEM-T) and the construct is named TMBC1m. The deposit has been assigned accession number ATCC No. 97244 by the repository.

The subject deposit was deposited under conditions that assure that access to the deposit will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 U.S.C. 122. The deposit will be available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject deposit will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., it will be stored with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposit, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the culture. The depositor acknowledges the duty to replace the deposit should the depository be unable to furnish a sample when requested, due to the condition of the deposit. All restrictions on the availability to the public of the subject culture deposit will be irrevocably removed upon the granting of a patent disclosing it.

To use the biological materials deposited, all that is necessary is for the DNA to be solubilized in an appropriate transformation buffer for the cell type into which the gene is to be transformed. For *E. coli*, competent cells are prepared and transformed according to methods well known in the art (see Maniatis *et al.*, 1982), and transformed cells selected in an ampicillin growth medium. The plasmid is then isolated from the *E. coli* and excised from the pGEM-T vector using, for example, HindIII restriction enzyme. The excised gene fragment has a size of about 1100 bp. The HindIII fragment is then cloned into the HindIII site of an appropriate expression vector as described below. In addition to the above, Figure 1 provides the sequence of the mutant gene of this invention, except for a stretch of 25 nucleotides corresponding to positions 1742-1766, which were not identified in initial sequencing efforts. There are several mutations in the polynucleotide of Figure 1. Those mutations are part of the instant invention. Further, Figure 2 provides the complementary strand of the mutant polynucleotide and shows the HindIII termini. Figure 3

provides the deduced amino acid sequence of the mutated gene product, except for amino acids 151-159 which were not identified in the initial sequencing. The differences in amino acid sequence between the wild-type BC1 product and mutant BC1 products are shown in Figure 9B. Figure 4 shows a comparison between the wild-type (upper sequence) and mutant protein (lower sequence) based on initial sequencing efforts.

While this description provides a specific gene and fragments thereof which confer resistance on plants to geminivirus and tobamovirus infection, those skilled in the art will recognize that mutations other than or in addition to the specific mutations shown herein could achieve similar results. In fact, the method taught herein, by which the mutant gene disclosed herein was obtained, is broadly applicable to the obtention of similarly useful mutated movement genes of any virus. Furthermore, it is predictable, based on the instant disclosure, that the instant genes and polynucleotide molecules described herein, as well as likewise-derived genes, can confer resistance on a plant against infection by a wide variety of plant pathogens which depend on movement gene or other gene products for their pathogenesis, including both DNA and RNA viruses.

Example 1 - Development of transgenic tobacco plants

A. Construction of BC1 gene into an expression vector. The BC1 gene (nucleotides between 1278 and 2311 of the B component of tomato mottle geminivirus; Abouzid *et al.*, 1992) was amplified from the extracts of tomato mottle geminivirus infected tomato plants by polymerase chain reaction (PCR) technology. The primers used to amplify viral BC1 were
5'-CCCAAGCTTCGAGTTCGAAACTGC-3' (SEQ ID NO. 1) and
5'-CCCAAGCTTAACGAAGTGTGTTTGAC-3' (SEQ ID NO. 2).
The amplified BC1 segment was cloned into a pGEM-T vector and then digested with Hind III. The excised BC1 segment was ligated into the unique Hind III site of the binary pKYLX 71:35 S^2 vector.

B. Agrobacterium transformation. Competent cells of *Agrobacterium tumefaciences* LBA 4404 were prepared as described by An, *et al.*, (1985). The BC1 gene in the pKYLX 71:35 S^2 vector was directly transferred into the *Agrobacterium*. The clone was kept in a -80° C freezer for further use.

C. Plant transformation. The *Agrobacterium* carrying the BC1 gene in the pKYLX 71:35 S^2 vector was used to transform the leaf discs of *Nicotiana tobacum* cv. Xanthi. The *Agrobacterium* cells were cultured in YEP broth containing 50 μg/ml kanamycin and 10 μg/ml tetracycline and 25 μg/ml streptomycin for 24-30 hours. *Agrobacterium* cells were collected and resuspended in YEP

broth. Leaf discs cut from expanded young sterile seedlings were dipped into the *Agrobacterium* suspension and then placed on a selective medium containing 200 μg/ml Mefoxin and 100 μg/ml kanamycin. Regeneration and selection were carried out with the media, and took 6-8 weeks. The kanamycin resistant plants were individually grown in soil under sterile condition for a week, and then transplanted to pots in a growth room and/or greenhouse.

Example 2 - PCR, Southern blot and ELISA analysis

Transformation of the tobacco plants was confirmed by PCR analysis for BC1 gene in chromosomal DNA extracts, by Southern blotting with a BC1 probe, and by ELISA analysis for NPT II (Neomycin Phosphotransferase II). Twenty-three plants were transgenic for BC1.

Example 3 - Western blot analysis

Infected leaves of tomato plants were powdered after freezing in liquid nitrogen and extensively ground with a mortar and pestle in two volumes of ice-cold grinding buffer (GB: 100 mM Tris-HC1, pH 8.0, 10 mM EDTA and 5 ml dithiothreitol)(Deom, *et al.*, 1990). Membrane and cell-wall fractions were prepared as described by Pascal, *et al.*, (1993). The blotting procedure was conducted essentially as described by Towbin, *et al.*, (1979) using a Bio-Rad Mini-Protein Electrophoresis Cell and Bio-Rad Trans-Blot Electrophoretic Transfer Cell. The separator gel for small proteins was prepared with 12.5% polyacrylamide in gel buffer (Laemmli, 1970). The protein gels were transferred to nitrocellulose membrane (Bio-Rad Trans-Blot, 0.4 μm). The detection of expressed BC1 protein in transgenic tobacco plants was conducted with Western-Light~Chemiluminescent Detection System (TROPIX, Inc.). The BC1 protein was detected at a relatively high level and extracts from about 50% of the plants showed a smaller (truncated) BC1 protein (28k Da) than the wild-type (33k Da).

Example 4 - Evaluation of transgenic tobacco plants for symptoms due to the expression of the BC1 gene

The BC1 gene has been implicated as a symptom inducing element of a bipartite geminivirus during infection. Eleven transgenic tobacco plants which expressed the full length BC1 protein showed disease symptoms. Twelve plants expressing the truncated BC1 protein did not show disease symptoms.

Example 5 – Resistance to tomato mottle geminivirus and tobacco mosaic tobamovirus

Transformed tobacco plants (R_1 generation) expressing BC1 were tested for susceptibility to tomato mottle geminivirus infection by natural transmission with the whitefly vector and by mechanical inoculation with extracts from infected plants. The inoculated plants were evaluated for resistance to tomato mottle geminivirus by symptom development, and by enzyme linked immuno-assays (ELISA) using antiserum reactive to tomato mottle geminivirus coat protein. The transgenic plants expressing the truncated BC1 protein were free of symptoms and had very low ELISA readings. Transgenic tobacco plants subjected to mechanical inoculation with tobacco mosaic tobamovirus showed reduced disease symptoms compared to inoculated non-transgenic plants.

Example 6 – Analysis of the BC1 gene expressing truncated protein

The BC1 gene from the tobacco plants expressing the truncated BC1 protein was PCR-amplified and sequenced. This data indicates that the BC1 gene has undergone spontaneous mutation(s) in about 50% of the transgenic BC1 tobacco plants. During the tissue culture phase, plant cells containing the mutated BC1 gene may have a selective advantage over the wild-type BC1 expressing cells.

Example 7 – Production of transgenic tomato plants

The mutated BC1 gene in the pKYLX 71:35 S^2 vector is suitable for the production of tomatoes transgenic for the gene via *Agrobacterium* transformation as described above for tobacco. The mutated BC1 gene provides similar resistance to tomato mottle geminivirus in tomato as seen in transgenic tobacco. The introduction of this mutated BC1 gene into the chromosome of desirable tomato lines leads to tomato mottle geminivirus resistance in commercially acceptable tomato cultivars/hybrids. In addition, it is predictable that this resistance is active against other geminivirus infections. Resistance to tobacco mosaic virus was also detected in the transgenic tobacco expressing the mutated BC1 gene, indicating that resistance to RNA viruses also is possible with the expression of this mutated gene from a DNA plant virus. The mutated gene in tomato offers resistance to tomato mosaic tobamovirus, a virus related to tobacco mosaic tobamovirus.

Example 8 – Production of BC1 gene fragments useful for conferring virus resistance to plants.

Fragments of the mutant BC1 gene which are useful for conferring virus resistance to plants can be produced by use of BAL31 exonuclease for time-controlled limited digestion of the mutant BC1 gene. Methods of using BAL31 exonuclease for this purpose are well known in the art, and have been widely used for over a decade (Wei *et al.*, 1983). By using BAL31 exonuclease, one can

easily remove nucleotides from either or both ends of the mutant BC1 gene to systematically and certainly generate a wide spectrum of DNA fragments which have controlled lengths and are from controlled locations along the entire length of the mutant BC1 gene. Hundreds of such fragments from various points along the entire mutant BC1 gene DNA sequence can be systematically generated in one afternoon. These gene fragments are then cloned into appropriate vectors and ultimately transferred into plant cells according to the methods disclosed above. Plant cells transformed with these fragments are routinely cultured and regenerated into plants, which are then tested for resistance to viruses. In this manner, fragments of the mutant BC1 gene which are sufficient to confer viral resistance are routinely and predictably identified.

Example 9—Production of additional mutants conferring virus resistance to plants.

Tobacco was transformed with the movement protein (pathogenicity) gene (BC1) from tomato mottle geminivirus (TMoV) using *Agrobacterium*-mediated transformation. Different transgenic tobacco lines expressing the BC1 protein had phenotypes ranging from plants with severe stunting and leaf mottling to plants with no visible symptoms. The sequence data for the BC1 transgene for the different phenotypes indicated unexpected mutation(s). A mutated BC1 transgene suppressed the phenotypic expression of the symptomatic BC1 gene in tobacco lines containing both copies of the BC1 gene. The present invention shows spontaneous mutations in the transgene to be common in *Agrobacterium*-mediated transformations, and this phenomenon can be utilized in the creation and selection of pathogen-resistant plants using pathogenicity genes during transformation.

The expression of tomato mottle gemini virus (TMoV) (Abouzid *et al.*, 1992) movement protein gene (BC1) was examined in transgenic tobacco plants for evaluation of function and for possible utilization in pathogen-derived resistance. The BC1 gene has been implicated as a symptom inducing element of a bipartite geminivirus during expression in transgenic plants (Pascal *et al.*, 1993; von Arnim and Stanley, 1992). Transgenic tobacco expressing the BC1 gene was constructed utilizing standard *Agrobacterium*-mediated transformation. Surprisingly, a number of plants expressing TMoV BC1 protein based on Western blot analysis, did not show the expected virus-symptom phenotype. Only 11 of the 19 transgenic R_0 tobacco plants which expressed the BC1 protein showed disease symptoms ranging from mild to severe. The observation that eight plants expressing the BC1 protein did not show symptoms was unexpected.

From further analysis, the three phenotypes were observed in the R_1 generation derived from a R_0 plant which did not show any apparent stunting (Fig. 5). The three observed phenotypes were: 1) Severe stunting and mottling, more severe than the typical symptoms associated with TMoV

infections in tobacco; 2) Mottling with no stunting of growth; and 3) No visible symptoms, plants indistinguishable from nontransformed plants.

These transgenic plants were analyzed by Southern blots to identify gene copy number (Fig. 6). The transgenic plant showing slight mottling with no stunting had two copies of the BC1 gene. Other progeny from this line which had a severe symptom phenotype or a non-symptomatic phenotype only had one copy. Progeny from three other lines examined, one with symptomatic and two with non-symptomatic phenotypes, had 3, 3, and 5 copies of the BC1 gene, respectively.

High levels of the BC1 protein expression were indicated in the young tissues in all transgenic plants by Western blot analysis except for one non-symptomatic line which showed low levels of a truncated BC1 protein. Non-symptomatic plant (BC1-3-6-4; phenotype not shown) had a similar level of BC1 protein as the symptomatic plant (BC1-3-11-5). Extracts from the attenuated-symptom plant (Fig. 5A and B; BC1-3-11-2) showed both full-length and truncated BC1 proteins. The low level of the truncated BC1 protein detection may be due to the loss of epitopes since 121 amino acid residues were lost at the carboxy end (see below). BC1 proteins (full-length or truncated form) from the non-symptomatic, transgenic plants were not detected in older tissue, unlike that seen for the transgenic plants expressing the severe symptom type BC1 protein. This indicated that certain mutations in the BC1 protein may affect its stability *in planta*.

Northern blots indicated a high transcript number for all the transgenic lines (Fig. 8). The non-symptomatic plant shown in Fig. 5A had a smaller than expected transcript. This apparent deletion in the transcript is consistent with the truncated BC1 protein seen in Western blots (Fig. 7). The transcript level for the plants expressing the truncated BC1 protein was high and therefore the low level of truncated BC1 protein detected in Western blots (Fig. 7) is not due to transcript activity. The larger than expected transcript is the result of a readthrough of BC1 termination signals into the vector *rbcS* termination sequences of the pKYLX vector.

The BC1 gene from the transgenic tobacco plants showing the different phenotypes was amplified by polymerase chain reaction (PCR) and sequenced. The sequence data revealed mutations (amino acid residue 215 G-S, 219 S-L, and 247 E-G) near the carboxyl terminus of the BC1 protein (Fig. 9) for the severe stunting phenotype (Fig. 5A). Two mutants were associated with non-symptomatic, transgenic tobacco. One mutant (resolved from BC1-3-6-3) showed several changes near the amino terminus (amino acid residue 6 V-F, 7 N-S, and 35 F-L) while the other (resolved from BC1-3-11-6, Fig. 5A) showed a change in amino acid residue 12 F-C, a deletion of amino acid residues 174-293, and an unidentified fusion sequence of 26 amino acid residues starting after amino acid residue 173 (Fig. 9). This was consistent with the detection of a truncated BC1 protein (~10 kDa smaller in size compared to the wild type) in Western blots from extracts from these transgenic

plants. The transcript for the truncated, BC1 protein was sequenced after reverse transcription of extracted total RNA using oligo dT primer followed by PCR amplification using a BC1 specific primer.

The non-symptomatic, transgenic R_0 tobacco plants revealed segregation in the R_1 generation as indicated by the appearance of several symptomatic plants in this generation. Some lines with symptom attenuation (Fig. 5B) continued to segregate in the R_2 generation but the non-symptomatic plants did not. Southern blot analysis (Fig. 6) indicated multiple copies of the BC1 gene in the R_0 tobacco. Apparently some of the R_0 tobacco lines contained copies of both the symptomatic and non-symptomatic forms of BC1. This was confirmed by Southern blot and Western blot analyses of selected R_1 tobacco plants which were associated with the different phenotypes (Fig. 5). The mottling phenotype with no stunting described above (Fig. 5) had one copy each of the symptomatic and non-symptomatic forms of BC1. Transgenic tobacco containing copies of both symptomatic and non-symptomatic forms of the BC1 gene (Fig. 5B) resulted in a nonstunting with mild mottling phenotype. This indicated that the non-symptomatic BC1 gene suppressed (trans-dominant negative interference) the symptom inducing element(s) of symptomatic BC1 gene in transgenic plants containing both forms. Transgene silencing (Meins Jr. and Kunz, 1995) was not evident in these plants since both proteins were detected in Western blots (Fig. 7, BC1-3-11-2). Furthermore, the expression of the symptomatic phenotype in subsequent generations indicated that the symptomatic BC1 gene was not in an inactive form in the phenotype suppressed R_0 tobacco. The symptom suppression was also effective against virus infection since tobacco plants with the mutated, non-symptomatic BC1 transgene remained free of TMoV symptoms under high disease pressure from viruliferous whiteflies over a 3-month period.

All of the BC1 transgenic plants that were analyzed revealed spontaneous/unexpected mutations in the BC1 gene. Point mutations were found in all transgenes analyzed, and one transgene showed a major deletion at the 3' end and with a fusion of an unidentified sequence of ~250 nucleotides (no close relationship with sequences in GenBank using BLAST). This may have occurred by a chromosomal cross-over event during plant cell division after the BC1 gene was integrated into the tobacco chromosome. In the latter case, a truncated BC1 protein (~10 kDa smaller in size compared to the wild type) was detected in Western blots and a smaller transcript was detected in Northern blots. This indicated that a deletion in the transgene as well as point mutations (described previously) are sources of variation in transgene expression.

Other studies with the expression of foreign genes in transgenic plants show varying levels of expressivity in the different generated lines or in siblings in a transgenic line (Hull, 1994). Varying levels of resistance in different transgenic plant lines transformed with the same gene appear

to be the norm in pathogen-derived resistance studies. These variations are not adequately explained by positional effects due to the random integration in the plant chromosome during transformation. Silencing of genes in transgenic plants is considered a general phenomenon when multiple copies of transgenes are introduced into plant cells (Meins Jr. and Kunz, 1995). All of our R_0 transgenic plants analyzed contained multiple copies of the BC1 transgene with no apparent suppression of transgene expression.

Because the teachings herein used the classical methods of *Agrobacterium*-mediated transformation commonly used by others in the art, some of the variation in the expected phenotype reported in the literature can be explained by spontaneous mutations occurring during *Agrobacterium*-mediated transformation and during chromosomal rearrangements as reported here for TMoV BC1. Thus it is shown that spontaneous point mutations in the transgene during *Agrobacterium*-mediated transformation and other modifications in the transgene by chromosomal rearrangements affect gene function and regulation with transgenes. The subject invention also concerns the polynucleotide molecules shown in Figure 9A and the polypeptides encoded thereby shown in Figure 9B, as well as other mutated polynucleotides conferring viral resistance that can be produced using the teachings of the present invention.

The spontaneous mutations that can be produced in viral movement genes using the methods and materials of the present invention during *Agrobacterium*-mediated transformation provide a simple way to develop pathogen-resistant plants. For geminiviruses, the introduction of the pathogenicity gene (BC1 for the bipartite geminiviruses, AC4 for the monopartite like tomato yellow leaf curl virus) into plant cells by *Agrobacterium*-mediated transformation will result in selection since transformed cells which express the non-mutated pathogenicity genes will not grow as well as those cells which express the mutated pathogenicity gene. After transformation, visual evaluation for the non-symptomatic phenotype and Western blot analysis for pathogenicity gene protein expression is all that is needed to select geminivirus-resistant plants for further screening and evaluation. All transgenic tobacco with a non-symptomatic phenotype and with TMoV BC1 protein expression showed virus resistance. Similarly, certain pathogenicity genes from bacterial or fungal plant pathogens can be introduced into transgenic plants according to these teachings and the selection pressure will result in pathogen-resistant plants.

The amino acid sequences disclosed herein are based on standard single letter abbreviations for amino acid residues.

While the foregoing description and examples provide details regarding the methods of making and using the invention, including its best mode, it is to be understood that obvious

variations and functional equivalents thereof are to be considered part of this invention and therefore fall within the scope of the claims which follow.

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Claims

1. A mutated plant virus BC1 gene which confers enhanced viral resistance to plants harboring said mutated gene.

2. The gene of claim 1 wherein the viral resistance is against a virus selected from the group consisting of tobamovirus and geminivirus.

3. The mutated gene of claim 1 prepared by the process of isolating the natural gene, inserting the natural gene into the genome of a plant, and identifying plants that have increased resistance to viral infection.

4. The mutated gene of claim 1 which encodes a gene product of about 28 kDa.

5. A method for conferring on a plant enhanced resistance against plant viral infection which comprises insertion of a viral movement gene into said plant and identifying a plant spontaneously expressing a mutant of said gene which confers said enhanced resistance against plant viral infection on said plant while at the same time not inducing pathogenic symptoms in the plant.

6. The method of claim 5 in which the viral movement gene is a plant virus BC1 gene.

7. A transgenic plant having increased resistance to viral infection, said plant being transgenic for a mutated plant virus BC1 gene.

8. The plant of claim 7 which is a transgenic tomato or tobacco plant.

9. The plant of claim 8 in which the plant has enhanced resistance against infection by tomato mottle geminivirus or tobacco mosaic tobamovirus.

10. The mutated gene of claim 1 which comprises any or all of the mutations as compared to the wild-type gene, shown in Figure 1 or Figure 9A.

11. The mutated gene of claim 1 comprising all or a portion of the sequence shown in Figure 1.

12. A mutant BC1 protein comprising any or all of the wild-type to mutant amino acid substitutions shown in Figure 4 or Figure 9B.

FIG. 1A

1278 TAA CGAAGIGIGT TIGACIAAAG

1301 ATGATTAACA TAAATGAAAA TGTAAAAATA AAATTTTATT TTAATGATTT

1351 CGICTGAGAC GCCTTACAAT TACTATTAAT ACATTCATGG ACCGTAGICC

1401 GTATTAATTC GTTCAACTGA CCCATAGACA TTGTAATGTT GGACTCTGCT

1451 TTCTGGGCCC CCACAATAGA AGCAGACTCT CCCGGGTCCA GTATGCCTGT

1501 TCCTAGCCTG TTTAGATGTC TGTACGGGTG GAGTTCGTTC TCCACTTCTG

1551 AGTCTGCATC TGAATGCCCT ATGCCTATTG TACTCCTTGA AGCCCATGAC

1601 TCACCAGGCC TGATCTCAAT TGGGCCTCTA AGCCCAAGTC TGGACATAGA

a

1651 CGCGCATCTA ATGGGCTTCC TCTCCCATTT ACCGTAATCC ACATGGGAAA

1701 AGTCCACATC TTTATCTGTG AACTGTTTGG ACAGGATTTT T+++++++++

1751 ++++++++++ ++++++TACT GAGTGTTTTG CTGTGGACAA TTTCAGCTTT

t

1801 CCCCTTAAAC TTGGCGAAGT GGGTCCGTTG ATGAACATTC GTATCGCAAA

1851 CCCTGTAATA CAATTTCCAT GGAATTGGGT CTTTCAAGGA GAAGAAGGAA

1901 GCTGAGAAAT AGTGGAGATC TATGTTGCAC CTGATCGGAA ATGTCCATGA

1951 TGCCTGTAAA GACTCATTCT CCGTCATCCT TTTGTCGTGA ATCTCCACTA

c

2001 TTACCGATCC AGTGGCGTTT ATTGGTACTT GTTGTCTGTA CTCTATGACG

2051 CAGTGGTCGA TTTTCATGCA GCTACGGCTG AGCCTAGCGG TTAACTGCGA

ac

2101 CGCCGTGGAC GGAAGTTGCA GTATTATCTC AGTTAGGTCA TGAGAAAGCT

t

2151 GATATTCGTC ACGGTGTGAC TCTATGTAGT TGAATGCACT AGGAGGACTA

c g

2201 AATAACTGAG AATCCATATA ATGAAAATAA GGCCGCGCAG CTGCAGTGAT

2251 TGCTGAAGTT GAATCAGAAA GAAGTCGAAC AAGCTATGAA ACGGCAGTTT

2301 CGAACTCGAA G

FIG. 2A

```
      TAACGAAGTGTGTTTGACTAAAGATGATTAACATAAATGAAAATGTAAAAATAAAATTTT
1278  --+---------+---------+---------+---------+---------+---------+------- 1337
      ATTGCTTCACACAAACTGATTTCTACTAATTGTATTTACTTTTACATTTTTATTTTAAAA

      ATTTTAATGATTTCGTCTGAGACGCCTTACAATTACTATTAATACATTCATGGACCGTAG
1338  --+---------+---------+---------+---------+---------+---------+------- 1397
      TAAAATTACTAAAGCAGACTCTGCGGAATGTTAATGATAATTATGTAAGTACCTGGCATC

      TCCGTATTAATTCGTTCAACTGACCCATAGACATTGTAATGTTGGACTCTGCTTTCTGGG
1398  --+---------+---------+---------+---------+---------+---------+------- 1457
      AGGCATAATTAAGCAAGTTGACTGGGTATCTGTAACATTACAACCTGAGACGAAAGACCC

      CCCCCACAATAGAAGCAGACTCTCCCGGGTCCAGTATGCCTGTTCCTAGCCTGTTTAGAT
1458  --+---------+---------+---------+---------+---------+---------+------- 1517
      GGGGGTGTTATCTTCGTCTGAGAGGGCCCAGGTCATACGGACAAGGATCGGACAAATCTA

      GTCTGTACGGGTGGAGTTCGTTCTCCACTTCTGAGTCTGCATCTGAATGCCCTATGCCTA
1518  --+---------+---------+---------+---------+---------+---------+------- 1577
      CAGACATGCCCACCTCAAGCAAGAGGTGAAGACTCAGACGTAGACTTACGGGATACGGAT

      TTGTACTCCTTGAAGCCCATGACTCACCAGGCCTGATCTCAATTGGGCCTCTAAGCCCAA
1578  --+---------+---------+---------+---------+---------+---------+------- 1637
      AACATGAGGAACTTCGGGTACTGAGTGGTCCGGACTAGAGTTAACCCGGAGATTCGGGTT

      GTCTGGACATAGACGCGCATCTAATGGGCTTCCTCTCCCATTTACCGTAATCCACATGGG
1638  --+---------+---------+---------+---------+---------+---------+------- 1697
      CAGACCTGTATCTGCGCGTAGATTACCCGAAGGAGAGGGTAAATGGTATTAGGTGTACCC
```

FIG. 2B

```
      AAAAGTCCACATCTTTATCTGTGAACTGTTTGGACAGGATTTTT++++++++++++++++
1698  --+---------+---------+---------+---------+---------+---------+------- 1757
      TTTTCAGGTGTAGAAATAGACACTTGACAAACCTGTCCTAAAAA++++++++++++++++

      +++++++++TACTGAGTGTTTTGCTGTGGACAATTTCAGCTTCCCCCTAAACTTGGCGA
1758  --+---------+---------+---------+---------+---------+---------+------- 1817
      +++++++++ATGACTCACAAAACGACACCTGTTAAAGTCGAAGGGGGATTTGAACCGCT

      AGTGGGTCCGTTGATGAACATTCGTATCGCAAACCCTGTAATACAATTTCCATGGAATTG
1818  --+---------+---------+---------+---------+---------+---------+------- 1877
      TCACCCAGGCAACTACTTGTAAGCATAGCGTTTGGGACATTATGTTAAAGGTACCTTAAC

      GGTCTTTCAAGGAGAAGAAGGAAGCTGAGAAATAGTGGAGATCTATGTTGCACCTGATCG
1878  --+---------+---------+---------+---------+---------+---------+------- 1937
      CCAGAAAGTTCCTCTTCTTCCTTCGACTCTTTATCACCTCTAGATACAACGTGGACTAGC

      GAAATGTCCATGATGCCTGTAAAGACTCATTCTCCGTCATCCTTTTGTCGTGAATCTCCA
1938  --+---------+---------+---------+---------+---------+---------+------- 1997
      CTTTACAGGTACTACGGACATTTCTGAGTAAGAGGCAGTAGGAAAACAGCACTTAGAGGT

      CTATTACCGATCCAGTGGCGTTTATTGGTACTTGTTGTCTGTACTCTATGACGCAGTGGT
1998  --+---------+---------+---------+---------+---------+---------+------- 2057
      GATAATGGCTAGGTCACCGCAAATAACCATGAACAACAGACATGAGATACTGCGTCACCA
```

FIG. 2C

```
     CGATTTTCATGCAGCTACGGCTGAGCCTAGCGGTTAACTGCGACGCCGTGGACGGAAGTT
2058 --+---------+---------+---------+---------+---------+---------+------- 2117
     GCTAAAAGTACGTCGATGCCGACTCGGATCGCCAATTGACGCTGCGGCACCTGCCTTCAA

     GCAGTATTATCTCAGTTAGGTCATGAGAAAGCTGATATTCGTCACGGTGTGACTCTATGT
2118 --+---------+---------+---------+---------+---------+---------+------- 2177
     CGTCATAATAGAGTCAATCCAGTACTCTTTCGACTATAAGCAGTGCCACACTGAGATACA

     AGTTGAATGCACTAGGAGGACTAAATAACTGAGAATCCATATAATGAAAATAAGGCCGCG
2178 --+---------+---------+---------+---------+---------+---------+------- 2237
     TCAACTTACGTGATCCTCCTGATTTATTGACTCTTAGGTATATTACTTTTATTCCGGCGC

     CAGCTGCAGTGATTGCTGAAGTTGAATCAGAAAGAAGTCGAACAAGCTATGAAACGGCAG
2238 --+---------+---------+---------+---------+---------+---------+------- 2297
     GTCGACGTCACTAACGACTTCAACTTAGTCTTTCTTCAGCTTGTTCGATACTTTGCCGTC

     TTTCGAACTCGAAG
2298 --+----------+- 2311
     AAAGCTTGAGCTTC
```

FIG. 3

```
  1  MDSQLFSPPS AFNYIESHRD EYQLSHDLTE IILQLPSTAS QLTARLSRSC
 51  MKIDHCVIEY RQQVPINATG SVIVEIHDKR MTENESLQAS WIFPIRCNID
101  LHYFSASFFS LKDPIPWKLY YRVCDINVHQ RTHFAKFRGK LKLSTAKHSV
151  +++++++++K ILSKQFTDKD VDFSHVDYGK WERKPIRCAS MSRLGLRGPI
201  EIRPGESWAS RSTIGIGHSD ADSEVENELH PYRHLNRLGT GILDPGESAS
251  IVGAQKAESN TTMSMGQLNE LIRTTVHECI NSNCKASQTK SLK*
```

FIGURE 4

```
  1 MDSQLVNPPSAFNYIESHRDEYQLSHDLTEIILQFPSTASQLTARLSRSC 50
    |||||..|||||||||||||||||||||||||:|||||||||||||||||
  1 MDSQLFSPPSAFNYIESHRDEYQLSHDLTEIILQLPSTASQLTARLSRSC 50

 51 MKIDHCVIEYRQQVPINATGSVIVEIHDKRMTENESLQASWTFPIRCNID 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 MKIDHCVIEYRQQVPINATGSVIVEIHDKRMTENESLQASWTFPIRCNID 100

101 LHYFSASFFSLKDPIPWKLYYRVCDTNVHQRTHFAKFKGKAEIVHSKTLS 150
    |||||||||||||||||||||||||||||||||||:||||||||||||||
101 LHYFSASFFSLKDPIPWKLYYRVCDTNVHQRTHFAKFRGKAEIVHSKTLS 150

151 RHSFRAPTVKILSKQFTDKDVDFSHVDYGKWERKPIRCASMSRLGLRGPI 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 ??????????KILSKQFTDKDVDFSHVDYGKWERKPIRCASMSRLGLRGPI 200

201 EIRPGESWASRSTIGIGHSDADSEVENELHPYRHLNRLGTGILDPGESAS 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 EIRPGESWASRSTIGIGHSDADSEVENELHPYRHLNRLGTGILDPGESAS 250

251 IVGAQKAESNITMSMGQLNELIRTTVHECINSNCKASQTKSLK* 294
    |||||||||||||||||||||||||||||||||||||||||||||
251 IVGAQKAESNITMSMGQLNELIRTTVHECINSNCKASQTKSLK* 294
```

FIG. 5B

FIG. 5A

FIG. 6

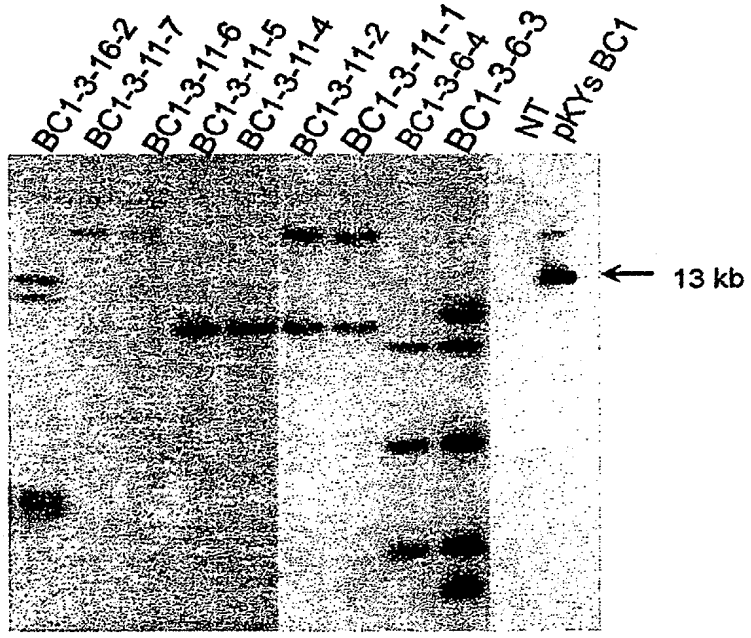

FIG. 7

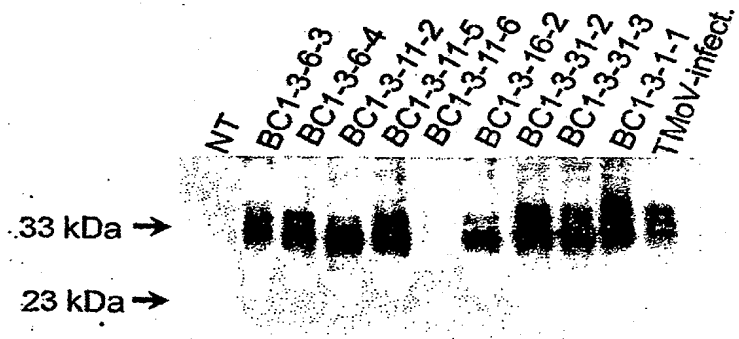

FIG. 8

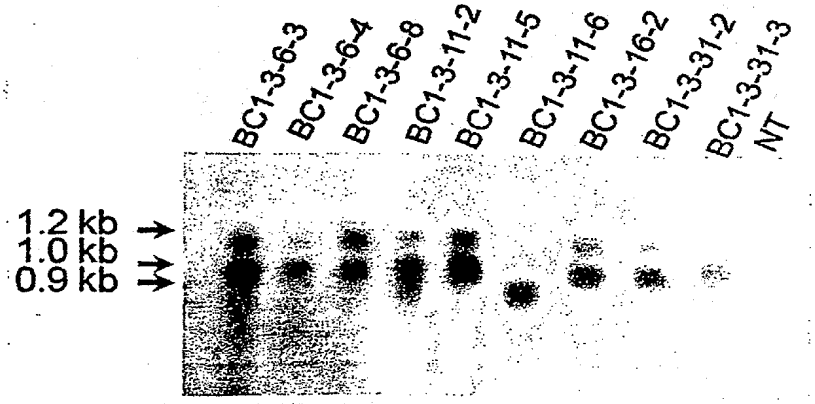

FIG. 9A-1

```
                1                                                    50
BC1wt            CGAGTTCGAA ACTGCCGTTT CATAGCTTGT TCGACTTCTT TCTGATTCAA
BC1S             .......... .......... .......... .......... ..........
BC1A             .......... .......... .......... .......... ..........
BC1scDNA         .......... .......... .......... .......... ..........
BC1At/rCDNA      .......... .......... .......... .......... ..........

               51                                                   100
               CTTCAGCAAT CACTGCCGCT GCGCGGCCTT ATTTTCATTA TATGGATTCT
               .......... .......... .......... .......... ..........
               .......... .......... .......... .......... ..........
               .......... .......... .......... .......... ..........
               .......... .......... .......... .......... ..........

               101                                                  150
               CAGTTAGTTA ATCCTCCTAG TGCATTCAAC TACATAGAGT CACACCGTGA
               .......... .......... .......... .......... ..........
               ......T... G......... .......... .......... ..........
               .......... .......... .......... .......... ..........
               .......... .......... .......... .......... ..........

               151                                                  200
               CGAATATCAG CTTTCTCATG ACCTAACTGA GATAATACTG CAGTTTCCGT
               .......... .......... .......... .......... ..........
               .......... .......... .......... .......... ......A...
               .......... .......... .......... .......... ..........
               .......... .......... .......... .......... ..........
```

FIG. 9A-2

```
201                                                          250
CCACGGCGTC  GCAGTTAACC  GCTAGGCTCA  GCCGTAGCTG  CATGAAAATC
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........

251                                                          300
GACCACTGCG  TCATAGAGTA  CAGACAACAA  GTACCAATAA  ACGCCACTGG
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........

301                                                          350
GTCGGTAATA  GTGGAGATTC  ACGACAAAAG  GATGACGGAG  AATGAGTCTT
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........

351                                                          400
TACAGGCATC  ATGGACATTT  CCGATCAGGT  GCAACATAGA  TCTCCACTAT
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
```

FIG. 9A-3

```
401                                                        450
TTCTCAGCTT CCTTCTTCTC CTTGAAAGAC CCAATTCCAT GGAAATTGTA
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........

451                                                        500
TTACAGGGTT TGCGATACGA ATGTTCATCA ACGGACCCAC TTCGCCAAGT
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........

501                                                        550
TTAAGGGGAA GCTGAAATTG TCCACAGCAA AACACTCAGT AGACATTCCC
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........

551                                                        600
TTCCGGGCAC CAACAGTAAA AATCCTGTCC AAACAGTTCA CAGATAAAGA
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
```

FIG. 9A-4

```
601                                                        650
TGTGGACTTT TCCCATGTGG ATTACGGTAA ATGGGAGAGG AAGCCCATTA
..........  ..........  ..T.....  ..........  ..........
..........  ..........  ..T.....  ..........  ..........
..........  ..........  ..T.....  ..........  ..........
..........  ..........  ..T.....  ..........  ..........
..........  ..........  ACCATTGAAA  CTGAGCCAAA  AATGCATGTT

651                                                        700
GATGCGCGTC TATGTCCAGA CTTGGGCTTA GAGGCCCAAT TGAGATCAGG
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
TTGGGGGAAA  ATTCCAAATA  GTGCAACTTT  TGAACAAACA  TACGATGGCT

701                                                        750
CCTGGTGAGT CATGGGCTTC AAGGAGTACA ATAGGCATAG GGCATTCAGA
..........  ..........  ..........  ...A......  .....T....
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ...A......  .....T....
AAAGTAGTAA  CAAGGCAAAG  GACGTAAACC  TAAAATGATG  TCCAAAAGCG

751                                                        800
TGCAGACTCA GAAGTGGAGA ACGAACTCCA CCCGTACAGA CATCTAAACA
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
TAATTATTCA  CTGTAGATGT  TTGTTTTACC  AACCAAAGAA  AAACTAAATT
```

FIG. 9A-5

```
801                                                          850
GGCTAGGAAC AGGCATACTG GACCCGGGAG AGTCTGCTTC TATTGTGGGG
.......... .......... .......... G......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... G......... ..........
GTGTATCCTA ATCAACAAAC TATCAGTAAG TTTCCAAATT ATTTCTAAAA

851                                                          900
GCCCAGAAAG CAGAGTCCAA CATTACAATG TCTATGGGTC AGTTGAACGA
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
AAAAAAAAAA .......... .......... .......... ..........

901                                                          950
ATTAATACGG ACTACGGTCC ATGAATGTAT TAATAGTAAT TGTAAGGCGT
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........

951                                                         1000
CTCAGACGAA ATCATTAAAA TAAAATTTTA TTTTTACATT TTCATTTATG
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........

1001                                   1031
TTAATCATCT TTAGTCAAAC ACACTTCGTT A
.......... .......... .......... .
.......... .......... .......... .
AAAAAAAAAA AAAAA..... .......... .
```

FIG. 9B

```
          1                                                  50
BC1wt     MDSQLVNPPS AFNYIESHRD EYQLSHDLTE IILQFPSTAS QLTARLSRSC
BC1A      .....FS... .......... .......... .....L.... ..........
BC1S      .......... .......... .......... .......... ..........
BC1At/r   .......... .......... .......... .......... ..........

         51                                                 100
BC1wt     MKIDHCVIEY RQQVPINATG SVIVEIHDKR MTENESLQAS WTFPIRCNID
BC1A      .......... .......... .......... .......... ..........
BC1S      .......... .......... .......... .......... ..........
BC1At/r   .......... .......... .......... .......... ..........

        101                                                 150
BC1wt     LHYFSASFFS LKDPIPWKLY YRVCDTNVHQ RTHFAKFKGK LKLSTAKHSV
BC1A      .......... .......... .......... .......... ..........
BC1S      .......... .......... .......... .......... ..........
BC1At/r   .......... .......... .......... .......... ..........

         151                                                200
BC1wt     DIPFRAPTVK ILSKQFTDKD VDFSHVDYGK WERKPIRCAS MSRLGLRGPI
BC1A      .......... .......... .......... .......... ..........
BC1S      .......... .......... .......... .......... ..........
BC1At/r   .......... .......... ....PLKLSQ KCMFWGKIPN SATFEQTYDG*

         201                                                250
BC1wt     EIRPGESWAS RSTIGIGHSD ADSEVENELH PYRHLNRLGT GILDPGESAS
BC1A      .......... .......... .......... .......... ..........
BC1S      .......... ....S...L. .......... .......... ......G...

         251                                          294
BC1wt     IVGAQKAESN ITMSMGQLNE LIRTTVHECI NSNCKASQTK SLK*
BC1A      .......... .......... .......... .......... ...*
BC1S      .......... .......... .......... .......... ...*
```

INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 96/13097

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/34 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 87, August 1990, WASHINGTON US, pages 6291-6295, XP002024195 STANLEY, J., ET AL.: "Defective viral DNA ameliorates symptoms of geminivirus infection in plants" see the whole document ---	1-4,7,8
X	PROCEEDINGS OF THE SYMPOSIUM MOL. BIOL. TOMATO, 1993, pages 227-238, XP000564182 DE KOUCHKOVSKY F., ET AL: "MOLECULAR BIOLOGY OF TOMATO YELLOW LEAF CURL VIRUS (TYLCV) AND POTENTIAL WAYS TO CONTROL THE DISEASE" see page 230 - page 233 ---	5,6

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[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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31 January 1997	11.02.97

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VIROLOGY, vol. 187, 1992, pages 555-564, XP002024283 VON ARNIM, A., ET AL.: "INHIBITION OF AFRICAN CASSAVA MOSAIC VIRUS SYSTEMIC INFECTION BY A MOVEMENT PROTEIN FROM THE RELATED GEMINIVIRUS TOMATO GOLDEN MOSIAC VIRUS" cited in the application see the whole document	1,2,4
Y	---	7-9
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, April 1993, WASHINGTON US, pages 3134-3141, XP002024284 WILSON, T.M.A.: "Strategies to protect crop plants against viruses: Pathogen-derived resistance blossoms" see page 3138, column 1	7-9
X	---	1-4,7-9
	VIROLOGY, vol. 206, no. 1, 10 January 1995, pages 307-313, XP002024285 COOPER, B., ET AL.: "A defective movement protein of TMV in transgenic plants confers resistance to multiple viruses whereas the functional analog increases susceptibility" cited in the application see the whole document	
X	---	10-12
	SWISSPROT PROTEIN SEQUENCE DATABASE. ACCESSION NO. P06001. 13-8-87, XP002024286 HOWARTH, A.J., ET AL.: "BL1 protein" see sequence & PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 82, 1985, WASHINGTON US, pages 3572-3576, HOWARTH, A.J., ET AL.:	
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	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, October 1994, WASHINGTON US, pages 10310-10314, XP002024287 BECK, D.L., ET AL.: "Disruption of virus movement confers broad-spectrum resistance against systemic infection by plant viruses with triple gene block" see the whole document	

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE PLANT CELL, vol. 5, July 1993, pages 795-807, XP002024288 PASCAL, E., ET AL.: "Transgenic tobacco plants expressing the geminivirus BL1 protein exhibit symptoms of viral disease" see the whole document	1-12
A	VIROLOGY, vol. 207, 20 February 1995, pages 191-204, XP002024289 INGHAM, D.J., ET AL.: "Both bipartite geminivirus movement proteins define viral host range, but only BL1 determines viral pathogenicity" cited in the application see the whole document	1-12
A	J GEN VIROL 73 (12). 1992. 3225-3229. , XP002024290 ABOUZID A M ET AL: "THE NUCLEOTIDE SEQUENCE OF TOMATO MOTTLE VIRUS A NEW GEMINIVIRUS ISOLATED FROM TOMATOES IN FLORIDA." see the whole document	1-12
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